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Regulation of Glucose Utilization in Tumors by a Stress-Modified Insulin: Anti-Insulin System[1]

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Contents

	Page
Introduction	352
Materials and Methods	353
Experiments and Observations	354
S91 Melanoma	354
Effect of Alloxan Diabetes	354
Effects of Temperature Stress	356
Mice with tumors of 1 to 3 grams	357
Mice with tumors of 5 to 20 grams	357
The Glycolytic Rate ($Q_{CO_2}^{N_2}$) Time Curve	359
Reversal of inhibition by insulin	360
Effects of stress, incubation temperature, and glucose concentration	360
Effect of Glucose Concentration	365
Percent insulin effect	365
Stress and glucose requirement	367
Effect of Steroids and Zinc	369
Testosterone and zinc	369
Sequential change of incubation temperature and effects of testosterone	371
Duration of stress and sensitivity to testosterone	375
Tumor Slices *versus* Homogenates	377
Nonmalignant Tissues and Temperature Stress	379
S91A Amelanotic Melanoma	381
Degree of Cell Variation	381
Effect of Stress, Testosterone, and Insulin on $Q_{CO_2}^{N_2}$	382
Krebs-2 Carcinoma	386
Ascites Tumor	386
$Q_{CO_2}^{N_2}$ time curve	386
Effect of stress and insulin	386
Effect of steroids	387

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Krebs-2 Carcinoma—Continued Page
Solid Tumor 388
Harding-Passey Melanoma 389
Magnitude of $Q_{CO_2}^{N_2}$ 389
Effect of Stress, Insulin, and Testosterone 390
Human Melanoma 392
Discussion 394
Role of Insulin in Tumor Metabolism 394
Insulin Action in Nonmalignant Tissues 395
Insulin:Anti-Insulin Hormones and Tumor Autonomy 396
Stress and Insulin:Anti-Insulin Systems 397
Mechanism of Insulin Action 399
Chemotherapeutic Implications 400
Possible Increase in Sensitivity to Oxygen Deprivation 400
Interaction with Acetylpodophyllotoxin-ω-Pyridinium Chloride 400
Summary 401
References 402

Introduction

When mice bearing melanoma S91 are exposed to high temperatures (35 to 40° C. for 12 to 20 hours) the *in vitro* anaerobic glycolysis ($Q_{CO_2}^{N_2}$) of slices of excised tumors is markedly lowered, but addition of insulin to such slices restores the rate of glycolysis to levels characteristic of tumors not exposed to the high temperatures (*1*). It is also notable that tumor growth is strongly inhibited upon continued exposure of the mice to 35 to 40° C. It was suggested on the basis of these studies [(*1*) p. 344] that the capacity of the tumor to maintain a high rate of anaerobic glycolysis might be dependent on the available supply of insulin, although such an ". . . apparent insulin deficiency of the heat-treated tumors (as indicated by insulin-reversible lowering of ($Q_{CO_2}^{N_2}$) may be the expression of an increased concentration of insulin-reversible glycolytic inhibitors such as have been reported in the pituitary and adrenals" (*2*). Later we reported (*3*) that certain sex-hormone steroids markedly inhibited *in vitro* glycolysis by melanoma S91 slices, and that under certain conditions this inhibition was also insulin-reversible. Furthermore, the effects of prior *in vivo* exposure to 35 to 40° C. and the *in vitro* effects of sex hormones showed considerable similarity and additivity.

The present paper reports further studies on the effect of exposing the host-tumor system to various temperatures upon *in vitro* anaerobic glucose metabolism, and related response to hormonal factors, in the Cloudman S91 melanoma (*4*), the derived Algire S91A amelanotic melanoma (*5*), the Krebs-2 carcinoma in both solid and ascitic forms (*6*), and the Harding-Passey melanoma (*7*). Data from a series of biopsies of one case of human melanoma are also presented. Techniques for *in vitro* metabolic analysis of tissue slices have been developed that provide information concerning the sensitivity of certain tumors to certain specifiable as well

as yet unidentified hormones. These techniques involve determinations of the kinetic forms of glycolytic time curves as affected by sequential changes in temperature of incubation, by varying glucose concentrations, and by differing sensitivities to exogenous insulin and steroids. It is shown that certain tumors may act as important bioindicators of a stress-modifiable insulin:anti-insulin hormonal system that affects glucose metabolism. It will be seen that the metabolic balance involved in the insulin:anti-insulin hormonal system is strikingly and reversibly affected *in vitro* by temperature over the range 0 to 45° C., insulin action in the balance being favored in general by higher temperature and anti-insulin action by lower temperature. The findings not only lend support to the view that metabolism and growth of certain tumors are strongly controlled by this stress-modifiable hormonal system, but also indicate that progressive cell modification can be associated with a progressive loss in hormonal sensitivity. Implications with respect to possible multiple chemotherapy are considered.

Materials and Methods

The mouse tumors employed were maintained by intramuscular transplantation, with the exception of the Krebs-2 carcinoma which was also grown in the ascites form by intraperitoneal injection. All the mouse tumors employed could grow in the BALB/c strain (hereafter referred to as C mice) as well as the DBA strain, but since the S91 tumors grew best in DBA mice and the other tumors grew equally well in either strain, the DBA strain was used chiefly. Human melanoma was obtained from several biopsies performed on 1 patient during 2 operative periods about 10 days apart.[4]

Methods of preparing tissue for study, media used for *in vitro* incubation of tissue slices, reagents used, etc., were as reported previously (*1*), or modified as described in the text. All zinc insulin[5] preparations were solubilized by dissolving the hormone in weak alkali (0.003 to 0.006 *M* NaOH or KOH) followed by immediate adjustment of the *p*H at 6.9 to 7.0, as described previously (*1*). Testosterone and other steroids were dissolved in 2 ml. of absolute ethanol followed by addition of 10 ml. of distilled water. Control vessels always received equivalent amounts of alcohol-water mixture minus steroid. The small amounts of alcohol present (*ca.* 0.7% final concentration) had no significant effect on glycolysis. All temperatures are in degrees centigrade.

Throughout this study tumor-bearing animals were exposed to various constant environmental temperatures, and, following such exposure, determinations were made *in vitro* of tumor slice or homogenate metabolism at several different controlled temperatures in various sequences in the same experiment (by transfer of manometers and vessels to previously

[4] The clinical aspects of these studies were made with the co-operation of Drs. Robert Smith, James Holland, and John Fahey.

[5] We are indebted to Drs. H. A. Clowes, Otto Behrens, and F. R. van Abeele, of Eli Lilly and Co., for all preparations of zinc insulin crystals (Lilly Lot No. 535664, 26 units/mg.) and amorphous insulin containing less than 0.01% zinc (Lilly Lot No. W-1302).

prepared thermostated baths). As will be indicated later, the *in vivo* effects of high, stressing temperature on tumor metabolism are presumably indirect, *i.e.*, by means of changes in host endocrine balance, since the rectal temperatures of mice so exposed were not markedly affected. On the other hand, during *in vitro* incubation of the tumor slices, the effect of temperature is clearly a direct one. In order to avoid possible confusion between these two types of temperature influence (indirect *in vivo* and direct *in vitro*), the following phraseology will be adhered to. In designating particular tumors, the temperature to which the tumor-bearing animal was exposed prior to *in vitro* experimentation will be indicated as a prefix, *e.g.*, "25° S91 melanoma," "35° S91A tumor," etc. On the other hand, the temperature of *in vitro* experimentation will be indicated simply as "temperature of incubation."

Anaerobic metabolic activities are expressed throughout as microliters of CO_2 (produced as a result of acid formation) per mg. of initial dry weight of tissue per hour ($Q_{CO_2}^{N_2}$). As reported previously (*1*) for melanoma slices, manometrically indicated acid can be accounted for under anaerobic conditions as lactic acid. Glucose determinations by the method of Somogyi (*8*, *9*) have shown that approximately 2 moles of CO_2 are released per mole of glucose disappearing from the incubation medium. The abbreviation KRBC will be used to designate Krebs-Ringer's solution plus 0.04 *M* $KHCO_3$, as previously (*1*). In references to the effects of insulin or testosterone it is understood that it is exogenous hormone (added *in vitro*) that is referred to. When the endogenous (intracellular) hormones are referred to, it will be specifically stated.

The conclusions arrived at in the present investigation are based on experiments usually repeated in one form or another many times. However, because of the complex and prolonged nature of much of the experimentation, the method of presenting in detail the results of single typical experiments has often been followed. In most cases, measurements of *p*H change in the incubation medium were made as a check against the manometric indications. Tumor-bearing mice were surface-disinfected with 70 percent ethanol before removing the tumor tissues, and precautions were taken throughout to avoid microbial contamination. Bacterial contaminations occurred only very rarely and were at once made evident by abnormal manometric readings and by phase-contrast microscopy of the vessel contents. The data from such experiments were discarded. $Q_{CO_2}^{N_2}$ values have been calculated to the second decimal place; however, such calculations are based on manometric readings taken to the nearest whole millimeter.

Experiments and Observations

S91 Melanoma

Effect of Alloxan Diabetes

If the *in vivo* temperature-induced (35 to 40° C.) lowering of glycolysis in S91 melanoma (*1*) is due simply to a drop in absolute endogenous

insulin concentration, a similar effect might be obtained by markedly lowering insulin secretion in mice maintained at lower temperatures. Accordingly, a number of S91 melanoma-bearing mice of the DBA strain were alloxanized by intravenous injection into the tail vein with 2 to 3 mg. of alloxan monohydrate freshly made up in distilled water at 0° C. The mice were maintained at 15 to 23° C. following injection. After 24 to 72 hours, the animals were sacrificed by decapitation and aliquots of blood collected for glucose determinations. $Q_{CO_2}^{N_2}$ values were determined at 37° C. in KRBC with 0.625 percent dextrose plus or minus zinc insulin, and were based on the average rates for 30 to 60 minutes of incubation. Table 1 summarizes the data, grouped according to host blood-sugar levels, from 21 melanoma-bearing mice in the normal glycemic range. Eight of these had received alloxan injections. Mice which escaped the effects of alloxan were considered as normal mice. The data from 15 hyperglycemic mice are presented in table 2. Above 390 mg. percent blood sugar there was an increase in the average percentage insulin effect (23 *versus* 10% average stimulation). Ten of the 11 mice involved had received alloxan injections. One exception (blood sugar 638) was presumably a "spontaneous" diabetic. While there is considerable variation within the two groups, application of Student's *t* test gives a probability (*P*) of less than 0.01 that the difference between the means is due to chance. In any case, the difference (23 *versus* 10%) is much less than that repeatedly observed when tumors from mice previously exposed to 35 to 40° C. (50% average insulin effect) were compared with

TABLE 1.—*Blood-glucose levels (normal range) and tumor* $Q_{CO_2}^{N_2}$ *values of 21 S91 melanoma-bearing DBA mice maintained at 15 to 23° C.*

Blood-glucose (*mg./100 ml.*)	$Q_{CO_2}^{N_2}$		Insulin effect (*percent*)	Sex	Treatment
	−Insulin	+Insulin			
77	12. 06	13. 24	10	♂	Control
84	12. 75	13. 79	8	♂	Alloxan
86	13. 14	14. 27	9	♂	"
90	11. 97	13. 14	10	♂	Control
91	14. 73	16. 31	11	♂	"
95	15. 18	16. 94	11	♂	Alloxan
96	8. 32	9. 86	19	♂	Control
100	16. 97	16. 73	−1	♂	"
100	13. 29	16. 77	26	♂	"
105	16. 02	16. 21	1	♂	"
108	12. 95	14. 74	14	♀	"
113	12. 79	13. 28	4	♀	Alloxan
114	11. 31	12. 37	9	♂	Control
114	12. 53	14. 95	19	♀	"
115	7. 43	9. 00	21	♂	Alloxan
126	11. 02	12. 82	16	♀	"
130	12. 33	11. 32	−8	♂	Control
132	9. 13	11. 29	24	♂	Alloxan
143	16. 47	18. 74	14	♂	"
145	15. 62	14. 55	−7	♂	Control
147	14. 48	16. 10	11	♂	"
Average	12. 88	14. 12	10		

TABLE 2.—*Blood-glucose levels (hyperglycemic) and tumor $Q_{CO_2}^{N_2}$ values of 16 S91 melanoma-bearing DBA mice maintained at 15 to 23° C.*

Blood-glucose (*mg./100 ml.*)	$Q_{CO_2}^{N_2}$		Insulin effect (*percent*)	Sex	Treatment
	−Insulin	+Insulin			
158	16. 41	19. 34	18	♂	Control
180	18. 62	19. 07	2	♂	"
187	14. 92	17. 50	17	♂	Alloxan
213	15. 52	16. 39	6	♂	"
Average	16. 37	18. 08	10		
396	8. 12	10. 26	26	♂	Alloxan
396	13. 59	17. 20	27	♀	"
400	13. 35	16. 54	24	♂	"
477	12. 09	15. 07	25	♂	"
500	13. 16	15. 36	17	♂	"
502	12. 53	12. 27	−2	♂	"
500+	9. 49	13. 03	37	♂	"
516	12. 05	15. 86	32	♂	"
560	9. 62	11. 41	19	♂	"
600+	12. 74	15. 56	22	♂	"
638	11. 02	15. 01	36	♂	Control
Average	11. 61	14. 32	23		

tumors from control mice (7% average insulin effect) maintained at lower temperatures [(*1*) p. 336]. It should be further noted that, while it might be anticipated that a lowered insulin secretion by the host would be associated with an increased *in vitro* sensitivity to insulin, the relationship may be only indirect. There can be no assurance that even the strongly diabetic mice completely lacked insulin. That is, the increased tumor sensitivity to insulin, which appears to be associated with pronounced alloxan diabetes in the host, may to a large extent reflect a stress-induced increase in the secretion of anti-insulin factors (*e.g.*, pituitary, adrenal, and/or gonadal hormones) rather than a lack of insulin *per se*.

Effects of Temperature Stress

Previously reported measurements of rectal temperatures of melanoma-bearing DBA mice exposed to different temperatures (*1*) made it unlikely that observed effects of such exposure on tumor growth and on the $Q_{CO_2}^{N_2}$ of tumor slices could be due to the direct action of temperature. These conclusions were based on the fact that the average rectal temperatures of mice bearing 1- to 3-gram melanomas, and maintained at 18 to 38° C., varied only within the range 34 to 39°. Furthermore, measurements of the direct effect of temperature on tumor slice glycolysis revealed no marked *in vitro* temperature-related differences in insulin sensitivity in the range between 35 to 40°.

Current studies have shown that insulin-reversible lowering of tumor slice $Q_{CO_2}^{N_2}$ can also be obtained under certain conditions following *in vivo* exposure to low temperatures (20° C. and below). Thus a temperature of

20° C., which is not stressful to mice with small tumors (1 to 3 gm.) becomes so when the mice bear very large tumors (5 to 20 gm.). In such cases, the animals are unable to maintain normal body temperature and are obviously in a state of severe stress. The *in vitro* glycolysis of such tumors is markedly sensitive to exogenous insulin. The various data obtained are consistent with the view that the anaerobic glucose metabolism of the S91 melanoma is highly sensitive to hormonal changes in the host brought on by exposure to stressful conditions.

Mice with tumors of 1 to 3 grams.—When mice bearing 1- to 3-gram tumors were exposed to 18 to 20° C., the mice remained active and body temperature was maintained in the range 34 to 39° C., while the tumor slice $Q_{CO_2}^{N_2}$ was at a high level with relatively low sensitivity to insulin (average stimulation only 9% in 33 tumors).

Mice with tumors of 5 to 20 grams.—When mice bearing very large S91 tumors (5 to 19 grams) were exposed to 20° C., the animals were unable to maintain normal body temperature for more than several hours. After 12 to 24 hours, the rectal temperatures fell to 20 or 21° C. and the animals were very sluggish. On the other hand, mice bearing similar large tumors, but exposed to temperatures of 25 to 28° C., or to 35° C., maintained body temperatures in the normal range, and the animals remained active. Tumors of 10 to 20 grams in weight, from mice maintained at 25 to 28° C., were much less sensitive to insulin than similar tumors from mice maintained at 35° C. (table 3; 11 *versus* 38% average stimulation). Blood sugars were significantly below normal in the 35° mice (average 56 mg. %). Of particular significance was the large insulin effect (40% average stimulation) in the 5- to 19-gram tumors from the 20° mice (table 3). The blood-sugar level in the 20° mice was significantly higher than in the 35° mice.

Considerable intratumoral variation in $Q_{CO_2}^{N_2}$ was often encountered in such large tumors, even when no visible necrotic areas were present. In most cases, representative samples were obtained by carefully mixing the sections from a large volume of tissue before weighing out aliquots, or various intratumoral regions were run separately. Both methods of sampling are illustrated in table 3. It can be seen that the differences in *percent* insulin effect (col. 8, table 3) between temperature treatments greatly exceeded those due to intratumoral variation, although, as would be expected, wide variations in absolute $Q_{CO_2}^{N_2}$ occurred. With mice bearing smaller tumors it can be readily shown that there is a significant insulin-reversible lowering of $Q_{CO_2}^{N_2}$ following exposure of the tumor-bearing animals to 35° C. (*1*).

The data accumulated to this point suggested that exposure of the host-tumor system to stress (low or high temperature) resulted in an increased *in vitro* sensitivity of the S91 tumor to insulin. This could have been the result of an increased endocrine secretion of anti-insulin glycolytic inhibitors by the host. Subsequent investigation showed that both the insulin-reversible lowering of tumor glycolysis and the inhibition of growth, which follow exposure of the host-tumor system to 35 to 40° C.,

TABLE 3.—*Effect of insulin on $Q_{CO_2}^{N_2}$, of massive S91 melanomas from DBA mice exposed to different temperatures*

Tumor number*	Weight (*grams*)	Temperature	Hours of exposure	Blood-glucose (*mg./100 ml.*)	$Q_{CO_2}^{N_2}$		Insulin effect (*percent*)	Condition of host
					−Insulin	+Insulin		
1	10	25–28° C.	continuous	75	8. 72	8. 52	−2	Active and warm
2 A	20	25–28° C.	continuous	75	6. 04	7. 25	20	"
2 B	20	25–28° C.	continuous	75	11. 84	13. 16	11	"
2 C	20	25–28° C.	continuous	75	10. 44	11. 16	7	"
2 D	20	25–28° C.	continuous	75	8. 26	9. 30	13	"
3	15	25–28° C.	continuous	75	16. 00	19. 00	19	"
Average	15	25–28° C.		75	10. 21	11. 39	11	
4	12	35° C.	18	65	6. 60	9. 00	36	Active and warm
5	10	35° C.	43	50	11. 84	15. 57	32	"
6	18	35° C.	21	35	11. 61	16. 13	39	"
7	10	35° C.	104	75	8. 53	12. 47	46	"
Average	13	35° C.	47	56	9. 64	13. 29	38	
8	13	20° C.	20	105	9. 91	14. 79	49	Very sluggish, rectal temp.=20–21° C.
9	18	20° C.	20	67	10. 33	14. 02	36	"
10	19	20° C.	20	147	14. 10	18. 61	32	"
11	5	20° C.	20	125	14. 91	20. 57	38	"
12 A	10	20° C.	20	140	9. 16	12. 78	40	(Very sluggish, cold to touch)†
12 B	10	20° C.	20	140	11. 40	17. 51	54	"
13 A	10	20° C.	19	100	8. 70	11. 86	36	"
13 B	10	20° C.	19	100	8. 56	12. 26	43	"
Average	13	20° C.	20	114	10. 88	15. 30	41	

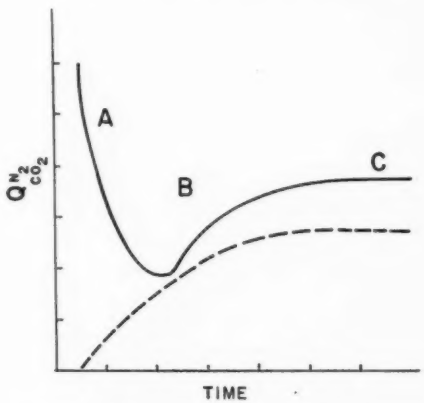
* Different regions of same tumor indicated by A, B, etc.

† Rectal temperatures not measured, but body temperatures obviously much lower than normal.

are tumor-specific phenomena. It is clear that there exists a definite spectrum of sensitivity to stress within the group of tumors studied. The experimental results bearing on these aspects of the problem will now be presented.

The Glycolytic Rate ($Q_{CO_2}^{N_2}$) Time Curve

When S91 melanoma slices were incubated anaerobically at constant temperature in a large volume of KRBC (to minimize changes in substrate concentration and *p*H) the $Q_{CO_2}^{N_2}$ values changed with time in a characteristic manner. The natures of the curves obtained were strongly influenced by (*a*) the extent of prior temperature stress of the host-tumor system, (*b*) the temperature of *in vitro* incubation (as well as the temperature of any previous *in vitro* incubation), (*c*) the glucose concentration, (*d*) the presence of exogenous insulin, (*e*) the presence of exogenous steroids (*e.g.*, testosterone but not cholesterol), and (*f*) other factors, such as the concentration of tissue per unit volume of KRBC, NaCl concentration, presence of zinc ions, etc. When incubation was carried out at low temperature (*e.g.*, 20° C.) the changes in $Q_{CO_2}^{N_2}$ occurred gradually, and, following establishment of glycolytic equilibrium, the rate was maintained for long periods (as long as 24 hours) with little further decay. While the nature of the curve obtained sometimes varied between tumors from different animals, certain general features could be recognized. Text-figure 1 is a conventionalized representation of the two main patterns of $Q_{CO_2}^{N_2}$ curves at 20° C. which characterize hormone-sensitive S91 melanomas. They are intended to illustrate only the most general forms of response, for individual variations in detail occurred. These variations



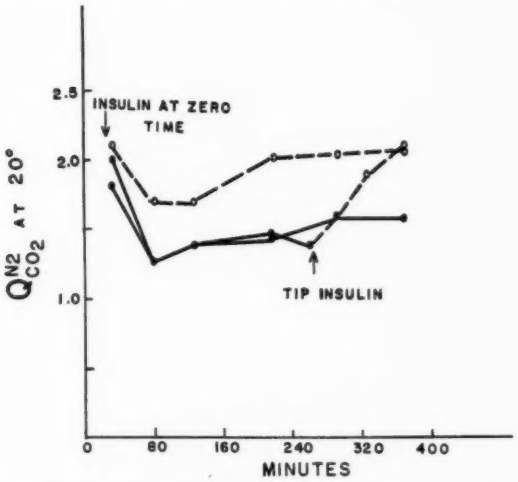
TEXT-FIGURE 1.—Schematic $Q_{CO_2}^{N_2}$ time curves for hormone-sensitive S91 melanoma slices incubated at 20° C. *Upper* curve (solid line) represents the usual pattern of response in which an initial phase of rapid decline (A) is followed by a period of variable recovery (B) and final attainment of glycolytic equilibrium (C); *lower* curve (broken line) represents the type of response sometimes obtained when the slices have been subjected to prolonged prior incubation at a low temperature or have come from a heavily stressed mouse. Note pronounced initial lag phase.

chiefly involved the degree of slope and the relative times required to reach a steady rate. Except in occasional tumors from very heavily stressed mice, or following prolonged previous incubation at a lower temperature, the curve usually resembled the form shown by the solid line in text-figure 1.

An initial phase of rapid decline (A) was followed by a period of recovery variable in rate (B), leading to establishment of glycolytic equilibrium (C) which was usually maintained with little further decline (3 to 24 hours) if adequately constant glucose and bicarbonate concentrations were maintained. Sometimes when the tumor slices were first incubated for a considerable period at a temperature below 20° C. (even 0° C.), and then suddenly transferred to a higher temperature (*e.g.*, 20 to 35° C.), the first phase appeared as in the broken-line curve of text-figure 1, indicating a pronounced but transient initial inhibition (lag phase) in glycolysis. The prior addition of insulin tended to reduce the extent of fall in phase A or tended to counteract the initial inhibition in tumors showing a lag phase. Furthermore, insulin accentuated the recovery phase and elevated the level of equilibrium. In many instances, the effect of insulin was maintained for over 24 hours; however, in some cases, especially in tumors from heavily stressed animals or in the presence of testosterone, a secondary fall to the control level without exogenous insulin occurred. Prior heat stress of the tumor-bearing mice accentuated the rate and extent of decline in phase A (text-fig. 1), tended to counteract the extent of recovery (phase B), and resulted in a lower level of equilibrium (phase C). The addition of certain steroids (*e.g.*, testosterone, *but not cholesterol*) affected the $Q_{CO_2}^{N_2}$ time curve in a manner resembling, but not necessarily identical with, that produced by prior heat stress. While saturation with testosterone markedly accentuated the tendencies toward glycolytic inhibition, it did not always equal the effects of heat stress alone. Furthermore, the potentiation of glycolytic inhibition by testosterone in tumors from heat-stressed mice was generally more pronounced percentagewise than in tumors from "unstressed" mice. Increasing the temperature of *in vitro* incubation, increasing the glucose concentration (*e.g.*, from 0.1 to 0.6%), and addition of insulin tended to counteract, separately and additively, glycolytic inhibition whether brought on solely by endogenous mechanisms or as a result of potentiation by exogenous steroid. The detailed results of several individual experiments will be presented to illustrate these varied aspects of behavior.

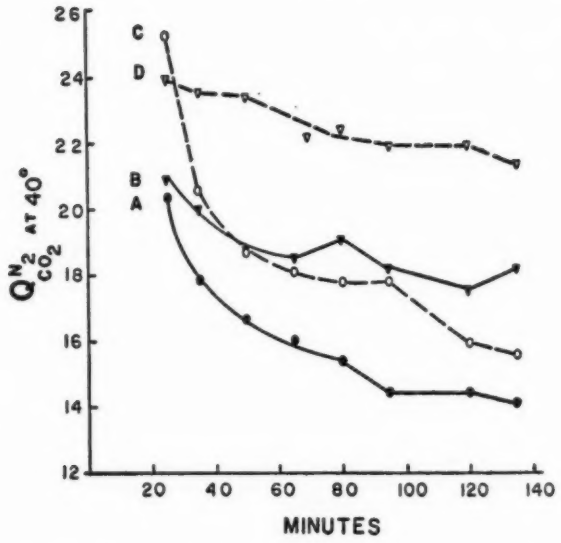
Reversal of inhibition by insulin.—The curves obtained at 20° C. with S91 melanoma slices from a mouse maintained under nonstressing temperatures (*ca.* 25° C.) are shown in text-figure 2. In this case, after 260 minutes of incubation at 20° C., addition of crystalline zinc insulin restored the glycolytic rate to that of tissue which had received an exogenous supply of insulin from the beginning of incubation. Similar results have been obtained in numerous experiments.

Effects of stress, incubation temperature, and glucose concentration.—Slices of two 32-day-old S91 melanomas from 2 strain DBA male mice of



TEXT-FIGURE 2.—$Q^{N_2}_{CO_2}$ time curves of S91 melanoma slices (washed 5 times by centrifugation in 20 ml. aliquots of KRBC plus glucose) from a strain DBA mouse maintained under nonstressing conditions (26° C.). The slices were transferred to 20° C., from 0° C., at zero time. Incubation was in KRBC plus 0.52 percent glucose (2.4 ml. final volume), and zinc insulin final concentration was 33 *gamma* (0.86 units) per ml., added at times indicated by arrows. Insulin was not added at all in the *lower* solid-line curve. Each vessel contained 200 mg. fresh weight of slices.

the same transplant series were compared following exposure of one to 35° C. for about 26 hours and the other to 25° C. for the same period. The tissue slices were incubated first at 40° C. for a period of 135 minutes and then at 20° C. for 80 minutes. Other experiments gave results which, though differing in detail, confirmed the main observations made in this experiment. Text-figure 3 shows the $Q^{N_2}_{CO_2}$ time curves at 40° C. for the 25° tumor at two levels of glucose (0.12 and 0.60% initial concentrations) in the presence and absence of zinc insulin. The curves for the 35° tumor are shown in text-figure 4. Corresponding curves following transfer to 20° C. are shown in text-figures 5 and 6. Table 4 compares the observed maximum and approximate equilibrium quotients for the two tumors under the four conditions of incubation at both temperatures. The correspondence in $Q^{N_2}_{CO_2}$ between the 25 and 35° tumors was maximal in the presence of high glucose and insulin (table 4, last column) at both incubation temperatures. The lowest degree of correspondence at both temperatures occurred with low glucose and in the absence of insulin. During approximate glycolytic equilibrium in low glucose at 40° C. (80 to 135 minutes, table 4), insulin increased the $Q^{N_2}_{CO_2}$ 15 percent in the 25° tumor and 77 percent in the 35° tumor. Corresponding values in the presence of high glucose were 20 percent and 38 percent, respectively. At 20° C. (50 to 90 minutes, table 4) in low glucose, insulin increased the $Q^{N_2}_{CO_2}$ of the 25° tumor only 7 percent, but gave an increase of 101 percent in the 35° tumor. Corre-



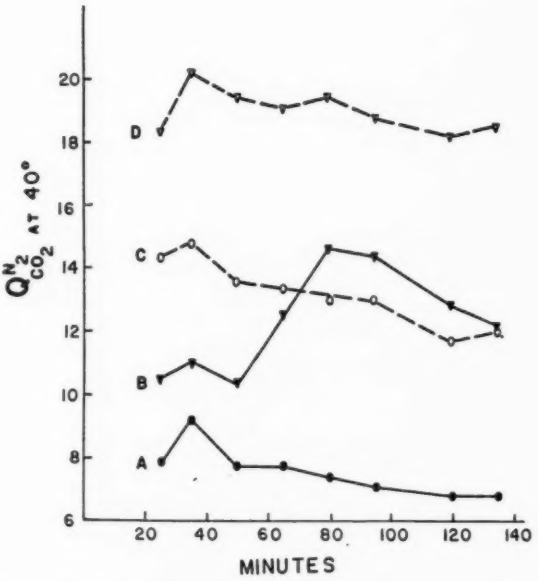
TEXT-FIGURE 3.—$Q^{N_2}_{CO_2}$ time curves for slices of a 25° S91 melanoma incubated at 40° C. in KRBC at two levels of glucose. A=control (minus insulin) with 0.116 percent initial glucose concentration; C=0.116 percent glucose plus 15 *gamma* (0.39 units) zinc insulin per ml.; B=control (minus insulin) with 0.60 percent initial glucose; D=0.60 percent glucose plus 15 *gamma* zinc insulin per ml. Total fluid volume was 5.2 ml.; 100 mg. wet weight of tissue per vessel.

sponding values in the presence of high glucose were 12 percent and 29 percent, respectively. It is thus clear that the greatest *percentage* effects of insulin occurred in the 35° tumor and at the lower concentration of glucose. Text-figure 3 indicates that the *initial* activity of the 25° tumor was about the same at both glucose concentrations in the presence of insulin (curves C and D), but the rate fell much more rapidly at the lower level of glucose (curve C). In the absence of insulin (curves A and B) the rate of decline was also greatest at the lower glucose level (curve A). It will be shown later that this type of progressive decline in $Q^{N_2}_{CO_2}$, down to an equilibrium level, is due mainly to factors other than progressive lowering of glucose concentration, although the latter becomes critical below about 0.05 percent at 40° C. The calculated lowest residual glucose concentration at the *end* of the 40° C. incubation cycle (text-fig. 3, curve C) was 0.056 percent. The factor of diminishing glucose concentration was of no consequence at the high (0.6% initially) glucose level (curves B and D). There were other marked differences between the curves for the two tumors. In the 35° tumor curves (text-fig. 4) there was a definite lag phase, and the effects of glucose concentration were more marked than in the 25° tumor. The forms of the curves are consistent with the postulate that during glycolytic equilibration (*cf.* text-fig. 1) the stress-intensified anti-insulin inhibitor mechanism at first tends to dominate the rate of

TABLE 4.—$Q^{N_2}_{CO_2}$, *values of 25° and 35° S91 melanomas at two temperatures of incubation and levels of glucose*

Incubation temperature (*period meas. in minutes*)	0.12 percent glucose				0.60 percent glucose			
	−Insulin		+Insulin		−Insulin		+Insulin	
	25° Tumor	35° Tumor	25° Tumor	35° Tumor	25° Tumor	35° Tumor	25° Tumor	35° Tumor
40°								
(25–35)	20. 23	7. 87	25. 27	14. 33	20. 93	10. 57	23. 96	18. 33
Equivalence*	.....	*39%*	.....	*57%*	.....	*51%*	.....	*77%*
(80–135)	14. 58	7. 01	16. 76	12. 41	18. 25	13. 55	21. 90	18. 72
Equivalence	.....	*48%*	.....	*74%*	.....	*74%*	.....	*85%*
20°								
(0–10)	6. 10	2. 73	6. 63	4. 93	5. 73	4. 33	6. 80	5. 57
Equivalence	.....	*45%*	.....	*74%*	.....	*76%*	.....	*82%*
(50–90)	2. 32	1. 00	2. 48	2. 01	2. 48	1. 90	2. 80	2. 40
Equivalence	.....	*43%*	.....	*81%*	.....	*77%*	.....	*86%*

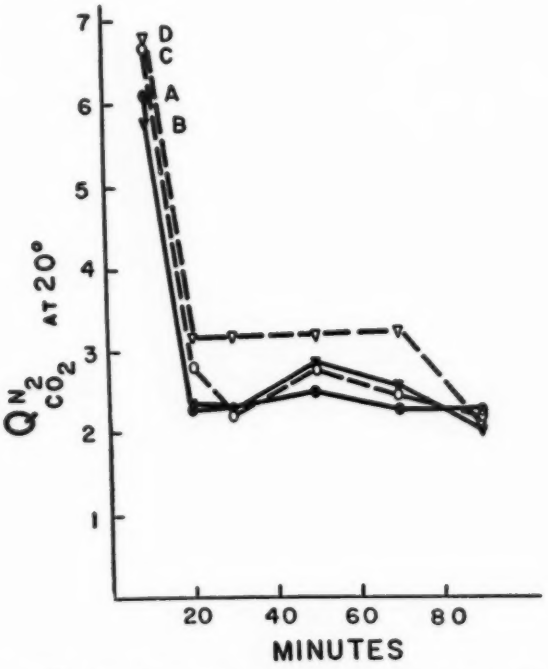
*35° Tumor activity as percent of 25° tumor activity.



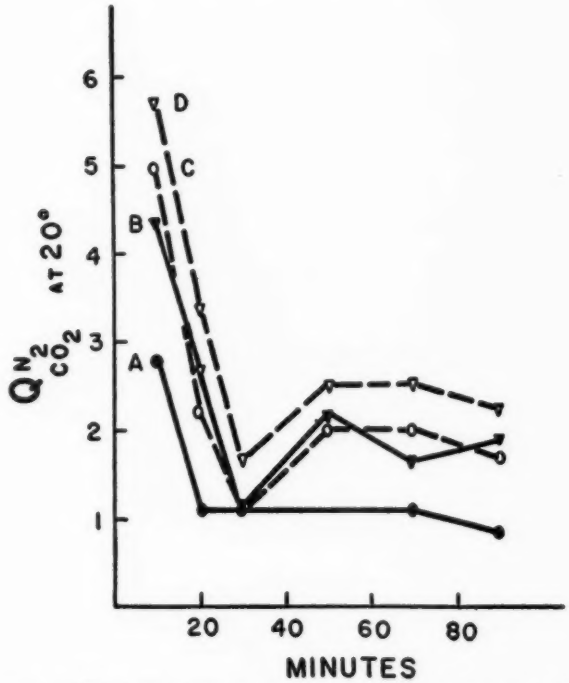
TEXT-FIGURE 4.—$Q^{N_2}_{CO_2}$ time curves for slices of a 35° tumor (26 hours' exposure) incubated at 40° C. in KRBC at two levels of glucose. A=control (minus insulin) with 0.116 percent initial glucose concentration; C=0.116 percent glucose plus 15 *gamma* (0.39 units) zinc insulin per ml.; B=control (minus insulin) with 0.60 percent initial glucose; D=0.60 percent glucose plus 15 *gamma* zinc insulin per ml. Total fluid volume was 5.2 ml.; 100 mg. wet weight of tissue per vessel.

glucose utilization, but in time the balance becomes progressively shifted toward insulin action (removal of inhibition). The secondary rise which may precede attainment of glycolytic equilibrium can be the result of a partial "catching up" by endogenous insulin. That is, the end points of inhibitor action and insulin action are reached at different times (noncompetitive inhibition). While the point will be discussed later in this paper, it should be noted that the anti-insulin inhibitor mechanism is almost certainly a multifactor one (*e.g.*, steroidal and nonsteroidal factors). On the basis of the foregoing postulates, curves A of text-figure 4 (low glucose) and B (high glucose) indicate that inhibitor action was slowly overcome in the presence of a high concentration of glucose, but not at the lowest glucose level. However, the addition of insulin to the low glucose medium (text-fig. 4, curve C) resulted in a much more rapid counteraction of inhibition than where the glucose level only was increased (curve B). Curve D shows that the effects of increasing glucose and insulin levels tended to be additive. A number of similar experiments have confirmed this type of interaction between glucose, insulin, and the endogenous inhibitor system.

When S91 melanoma slices which had been incubated at a high temperature (*e.g.*, 40° C.) were transferred to a lower temperature (*e.g.*, 20°



TEXT-FIGURE 5.—$Q^{N_2}_{CO_2}$ time curves for same tissue as in text-figure 3 but following transfer to 20° C. Final *p*H values were determined after 905 minutes of incubation at 20° C.



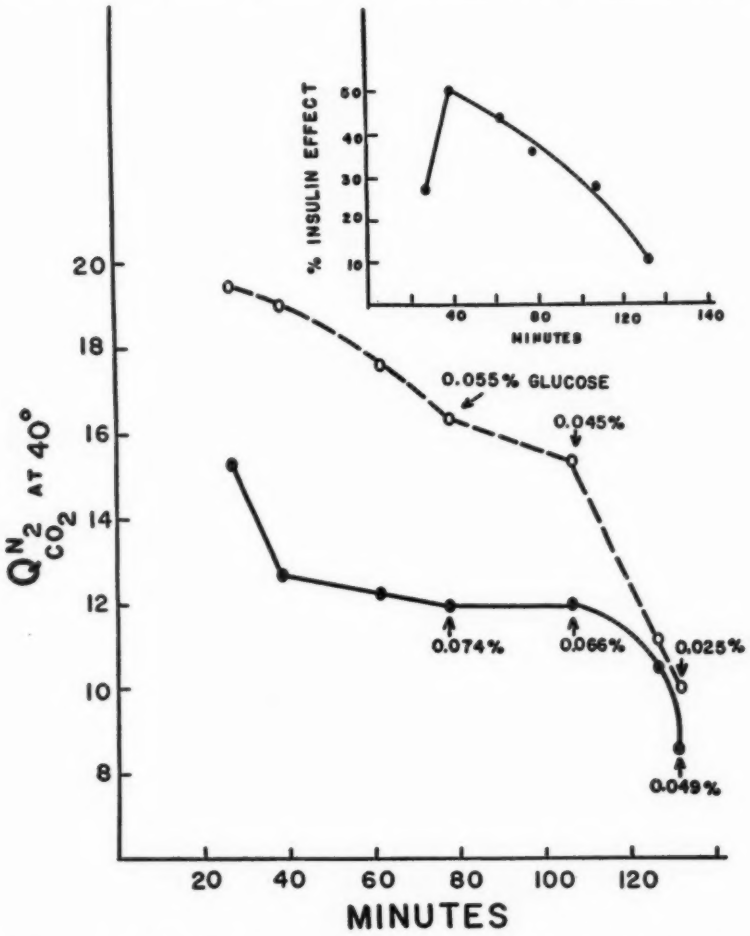
TEXT-FIGURE 6.—$Q^{N_2}_{CO_2}$ time curves for same tissue as in text-figure 4 but following transfer to 20° C. Final *p*H values were determined after 905 minutes of incubation at 20° C.

C.), $Q^{N_2}_{CO_2}$ *changed in time, as would be expected if a readjustment in balance between anti-insulin factors and insulin had taken place.* This is illustrated by text-figures 5 and 6 which show the $Q^{N_2}_{CO_2}$ time curves at 20° C. for the slices previously incubated at 40° C. (text-figs. 3 and 4). As at 40° C., addition of insulin, and/or high glucose, tended to poise glycolysis at a higher level. This was most clearly shown in the 35° tumor (text-fig. 6) where, following an initial fall in $Q^{N_2}_{CO_2}$ to a minimum value, there was a rebound to a higher level of approximate equilibrium in all cases except in low sugar minus insulin (A, text-fig. 6). However, in the presence of insulin there was a rebound even at the low level of glucose (C, text-fig. 6). With the 25° tumor (text-fig. 5) the initial drop was less pronounced, and there was less difference between glucose levels. As at 40° C., the differences in $Q^{N_2}_{CO_2}$ of the two tumors were minimal in the presence of high glucose and insulin (text-figs. 5 and 6, D curves).

Effect of Glucose Concentration

Percent insulin effect.—S91 melanomas taken from temperature-stressed mice and incubated *in vitro* at 35 to 40° C. in KRBC plus 0.6 percent glucose have shown an average stimulation of glycolysis by insulin of

about 50 percent. Similar tumors from "unstressed" animals under the same conditions of *in vitro* incubation have shown an average insulin effect of only 10 percent. However, it was observed that if "unstressed" S91 melanoma-bearing mice were killed and the rapidly excised tumor tissue, together with adhering blood, was very quickly transferred to ice-cold, glucose-free KRBC and gassed at 0° C., a transitory production of acid sometimes occurred following transfer to 30° C., presumably at the expense of endogenous glucose. The addition of zinc insulin greatly



TEXT-FIGURE 7.—$Q^{N_2}_{CO_2}$ time curves (incubation at 40° C.) for slices (200 mg. wet weight per vessel) of a 25° S91 melanoma in KRBC with an initial glucose concentration of 0.12 percent (5.2 ml.). Zinc insulin concentration was 17 *gamma* (0.44 units) per ml. Calculated residual glucose concentrations (see text) are indicated on the curves. Broken-line curve is with insulin; solid-line curve is without added insulin. The inset (*upper right*) shows the percentage stimulation by insulin in time.

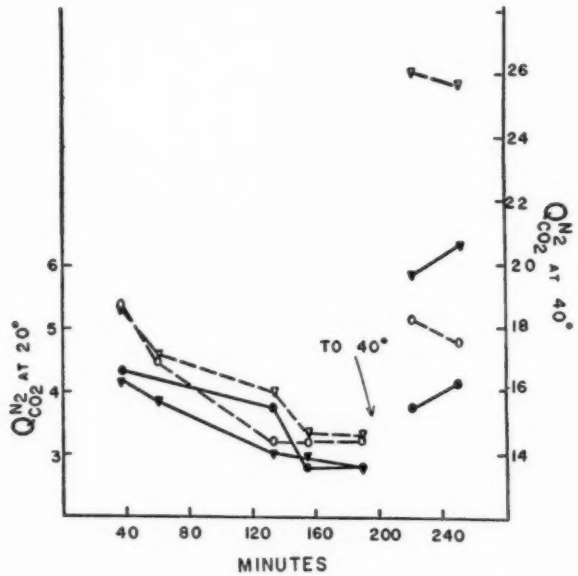
increased the rate of this acid production for a brief time, sometimes yielding insulin effects of several hundred percent. Results of this type suggested that if a low but relatively constant level of glucose could be maintained, large insulin effects might be obtained even in tumors from animals that had not been exposed to temperature stress. The results of such an experiment are shown in text-figure 7.

Final glucose determinations showed that approximately 2 moles of CO_2 were produced per mole of glucose removed from the medium. The calculated glucose concentrations following various times of incubation are indicated on the curves (text-fig. 7). The data of this and of other similar experiments indicated that the initial changes in rate were due largely to equilibration of the inhibitor-insulin complex and were not the result of falling glucose concentration. However, a final, sharp, secondary fall (after 100 minutes, in text-fig. 7) occurred after a critical concentration of glucose had been reached (*ca.* 0.066 % in the absence of exogenous insulin and about 0.045% with insulin). The secondary fall was definitely not the result of a lowered *p*H, since adequate buffer capacity was present at all times. The critical levels of glucose varied somewhat between tumors but were of the same order of magnitude as those shown in the text-figure. When the percentage insulin effect was calculated from $Q^{N_2}_{CO_2}$ values (text-fig. 7) *at equal glucose concentrations* (range 0.095 to 0.049%) the average insulin effect was 56 percent, being nearly linear throughout the time period. However, this is not an entirely valid procedure, since changes in rate with time that are not primarily the result of changing glucose concentration are involved. When the percent insulin effect was plotted for *corresponding points in time* (insert, text-fig. 7) a maximum stimulation of 50 percent was obtained at 40 minutes, but this fell to about 10 percent by 132 minutes.

Stress and glucose requirement.—In "unstressed" animals at low incubation temperatures (*e.g.*, 20° C.), where the rate of glucose metabolism was low, there was sometimes little difference in the $Q^{N_2}_{CO_2}$ values of slices from the same tumor at both low (0.1%) and high (0.6%) glucose concentrations. However, when the *in vitro* temperature was markedly increased, glucose concentration immediately became limiting. In the experiment illustrated in text-figure 8, increasing the incubation temperature from 20 to 40° C., increased the rate of glycolysis 453 and 465 percent in low glucose without and with insulin, respectively. At the high glucose level these increases were 625 and 703 percent, respectively. Chemical determinations at the end of the experiment showed residual glucose concentrations of about 0.08 and 0.54 percent, respectively, in the low and high sugar series. The individual values for each vessel were in satisfactory agreement with the formation of 2 moles of CO_2 per mole of glucose utilized.

A large number of experiments clearly demonstrated that the capacity of S91 melanomas to utilize glucose *in vitro* at low concentrations (0.1% or less), even more so than at high concentrations, was strongly influenced by the degree of prior temperature stress of the host-tumor system. This is

shown in table 5, in which the $Q_{CO_2}^{N_2}$ values of tumors from mice stressed for different lengths of time at 20° C. in the presence of 0.048 percent or 0.60 percent glucose concentrations are compared. During the first time period (16 to 36 minutes), both tumors in the presence of high glucose had a $Q_{CO_2}^{N_2}$ of about 2.0 with little response to insulin (plus or minus 10%). However, at the low glucose level, $Q_{CO_2}^{N_2}$ in both cases was only one half of the values obtained at the high glucose level. Insulin completely counteracted the inhibitory effect of low glucose in the tumor from the mouse stressed just 16 hours (201% insulin effect), but did so only slightly in the tumor from the animal stressed 68 hours (12% insulin



TEXT-FIGURE 8.—$Q_{CO_2}^{N_2}$ time curves for slices (100 mg. wet weight per vessel) of a 25° S91 melanoma at two levels of glucose (0.1 *versus* 0.6% initial concentrations; total volume 5.2 ml.), incubated first at 20° and then at 40°. Zinc insulin concentration was 15 *gamma* per ml. Circles=0.1 percent initial glucose; triangles=0.6 percent initial glucose. Solid lines are without insulin, broken lines are with insulin.

effect). During the 66- to 81-minute interval (table 5) the $Q_{CO_2}^{N_2}$ values in the presence of high glucose declined markedly in the 68-hour tumor but not in the 16-hour tumor. The low glucose values during the same period showed that where the factors tended to favor inhibition (longest period of stress, low glucose) glycolytic equilibrium was more rapidly attained, and exogenous insulin exerted little delaying action (14% effect). On the other hand, in the 16-hour tumor with low glucose there was still a pronounced insulin effect (39%), although the magnitude of the effect had been markedly reduced and equilibration was less complete. The results of this and other experiments (*e.g.*, text-figs. 5 and 6), showed

TABLE 5.—*Length of time of temperature stress in relation to* in vitro *glucose utilization by S91 melanoma*

Time of incubation at 20° C. (*minutes*)	$Q^{N_2}_{CO_2}$ of tumor from mouse stressed 16 hrs. at 35° C.				$Q^{N_2}_{CO_2}$ of tumor from mouse stressed 68 hrs. at 35° C.			
	0.048% Glucose		0.600% Glucose		0.048% Glucose		0.600% Glucose	
	−Insulin	+Insulin	−Insulin	+Insulin	−Insulin	+Insulin	−Insulin	+Insulin
16–36	0. 95	2. 86	2. 23	2. 01	0. 85	0. 95	2. 01	2. 23
% Insulin effect	.....	*201%*	.....	*−10%*	.....	*12%*	.....	*11%*
66–81	1. 23	1. 71	1. 99	1. 99	0. 94	1. 07	1. 54	1. 45
% Insulin effect	.....	*39%*	.....	*0%*	.....	*14%*	.....	*−6%*

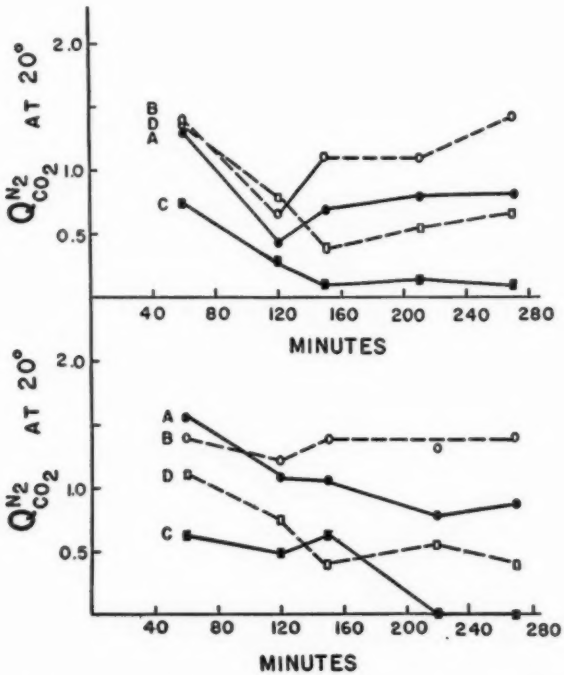
that *prior exposure to temperature stress lowered the capacity of the tumor to utilize low concentrations (0.05 to 0.10%) of glucose* in vitro. It should be noted that in tumor slices from mice exposed to severe stress, exogenous insulin was sometimes unable to overcome the endogenous inhibiting action, especially at low incubation temperatures. Such a condition is illustrated in table 5 by the tumor slices from the mouse stressed at 35° C. for 68 hours where the Q values after 66 minutes' incubation were markedly lower than in the tumor slices from the mouse stressed for only 16 hours. In such cases the percent insulin effect, as well as the glycolytic rate in the presence or absence of insulin, was actually lowered as the result of "overstress," this being particularly marked at low incubation temperature and low glucose concentration. The *in vitro* data suggest that levels of anti-insulin hormones and insulin may be critical in relation to the circulating concentration of glucose in determining survival and growth of tumor cells *in vivo*. The possible interrelation of these factors with oxygen tension will be discussed later.

Effect of Steroids and Zinc

Insulin-reversible inhibition of melanoma glycolysis by sex steroids [(*3*) and unpublished data] and by adrenocortical steroids (unpublished) has indicated that part of the endogenous (intratumoral) glycolytic inhibitor mechanism involves a steroidal factor(s). In the present study, the *in vitro* action of testosterone was employed as a tool to further delineate the nature of this mechanism. Inorganic zinc was found to play a significant role in this connection.

Testosterone and zinc.—The addition of testosterone (*ca.* 70 *gamma* per ml.) to the incubation medium resulted in a marked lowering of the $Q^{N_2}_{CO_2}$ of S91 melanoma slices. The inhibitory action of testosterone was usually, but variably, counteracted by the simultaneous addition of insulin. The addition of ionic zinc tended to accelerate testosterone-induced inhibition, although the final level of glycolytic equilibrium was sometimes even slightly higher than without zinc.

Text-figure 9 shows (lower group of curves) the $Q^{N_2}_{CO_2}$ time curves for slices of a 35° S91 melanoma (44 hours' stress) in KRBC plus 0.50 percent glucose.



TEXT-FIGURE 9.—*Lower group of curves.*—$Q^{N_2}_{CO_2}$ time plots at 20° C. for slices (200 mg. wet weight per vessel) of a 35° S91 melanoma (44 hours' stress) in KRBC with 0.5 percent initial glucose (6.2 ml. total volume). A=control (minus insulin); B=plus 20 *gamma* low-zinc insulin per ml.; C=plus 65 *gamma* testosterone; D=plus 20 *gamma* (0.5 unit) low-zinc insulin and 65 *gamma* testosterone per ml. *Upper group of curves.*—Same tumor, conditions, and time of incubation as in lower group, but all vessels contained 2.4 *gamma* of ionic zinc per ml.

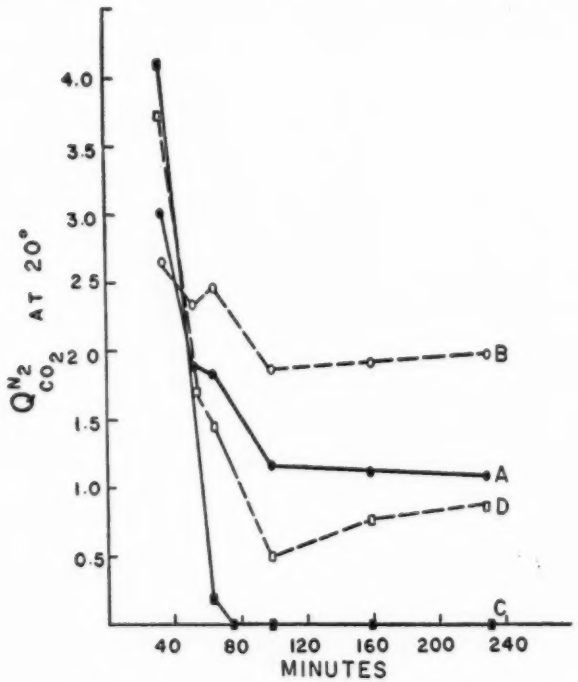
Low-zinc insulin (less than 0.01% Zn) was added to give a final concentration of about 20 *gamma* (0.5 unit) of insulin per ml. in a total fluid volume of 6.2 ml. Inorganic zinc (as $ZnSO_4 \cdot 7H_2O$) was added to one set of vessels (upper group of curves) to give a final concentration of 2.4 *gamma* Zn per ml., which was about 1.8 times the stoichiometric equivalent for the nearly zinc-free insulin present. Sixty-five *gamma* per ml. of testosterone was added. The initial (0 to 30 minutes) $Q^{N_2}_{CO_2}$ values were not recorded, but it can be seen (lower curves) that at approximate equilibrium (220 minutes), the inhibition, in the presence of testosterone, but in the absence of insulin (curve C), was 100 percent. On the other hand, in the presence of low-zinc insulin the inhibition was only 67 percent (curve D). The addition of inorganic zinc apparently affected the endogenous in-

hibitor mechanism (compare A and B, upper and lower curves) as well as the inhibition induced by testosterone (compare C and D, upper and lower curves). This was evidenced by the more rapid fall to minimum $Q_{CO_2}^{N_2}$ values, where exogenous zinc was present (upper curves). The data of this and similar experiments indicated that inorganic zinc was playing at least a dual role. It accentuated glycolytic inhibition during the earlier phase of the $Q_{CO_2}^{N_2}$ time curve (compare A, upper and lower curves) but had little effect on the final point of equilibrium, except possibly to elevate it slightly. In a similar manner, zinc accentuated testosterone-induced inhibition (compare C, upper and lower curves). Contrariwise, zinc sometimes exerted a stimulating action, presumably by enhancing the efficacy of the low-zinc insulin itself (*1*). The stimulating effect of zinc, which sometimes occurred in the presence of testosterone, was evidenced in the present experiment by the fact that, in spite of the more precipitous initial decline in $Q_{CO_2}^{N_2}$ (C curves), the final equilibration was at a higher level where zinc had been added. This was evident even in the presence of exogenous insulin (D curves), although in this case it would appear that the zinc-induced accentuation of the initial rate of decline in $Q_{CO_2}^{N_2}$ was more than offset by the stimulating action of zinc.

Sequential change of incubation temperature and effects of testosterone.— Text-figure 10 shows the $Q_{CO_2}^{N_2}$ time curves for a 35° S91 melanoma (19 hours' stress) incubated at 20° C. All vessels contained zinc at a concentration of 2.5 *gamma* per ml. While the initial $Q_{CO_2}^{N_2}$ values at 20° C. in the presence of testosterone (C and D, text-fig. 10) were higher than in the controls without testosterone (curves A and B), this situation was quickly reversed. Most notable was the rapid attainment of 100 percent inhibition of glycolysis in the presence of testosterone (C, text-fig. 10). This inhibition persisted for over 160 minutes. Insulin (D, text-fig. 10) markedly counteracted the testosterone-induced inhibition, although there was a sharp drop initially. Following transfer to 35° C. (text-fig. 11), testosterone-treated tissue, in which $Q_{CO_2}^{N_2}$ had previously been reduced to zero (C, text-fig. 11), showed a marked reactivation (C, text-fig. 11) to a rate nearly equal to that of the insulin-treated tissue (D, text-fig. 11). However, 25 minutes later the rate (curve C) had fallen sharply again, whereas the tissue treated with *both* insulin and testosterone remained at the level of the control without testosterone (curve D). With elevation of the incubation temperature still further to 45° C., $Q_{CO_2}^{N_2}$ was markedly increased in all cases. This time the curve for the testosterone-treated tissue (C, text-fig. 11) did not show a secondary fall. In fact it resembled the 20° curve for tissue treated with *both* testosterone and insulin (D, text-fig. 10). Whereas at 35° C. the $Q_{CO_2}^{N_2}$ with testosterone plus insulin (D, text-fig. 11) was about equal to the control $Q_{CO_2}^{N_2}$ (A, text-fig. 11), this was not the case at 45° C. where the control (A) was higher than D. Restated in another way, it can be said that raising the temperature of incubation resulted in a marked counteraction of glycolytic inhibition caused by the endogenous inhibitor mechanism, or to a lesser extent, by this mechanism plus testosterone. This resulted in a marked lowering

of the percent insulin effect at the higher temperatures (text-fig. 11, 45 and 50° C.). The very pronounced decay above 50° C. was probably the result of thermal inactivation. It was not due to lowering of the *p*H, which was within the range 7.08 to 7.39 at the end of the experiment.

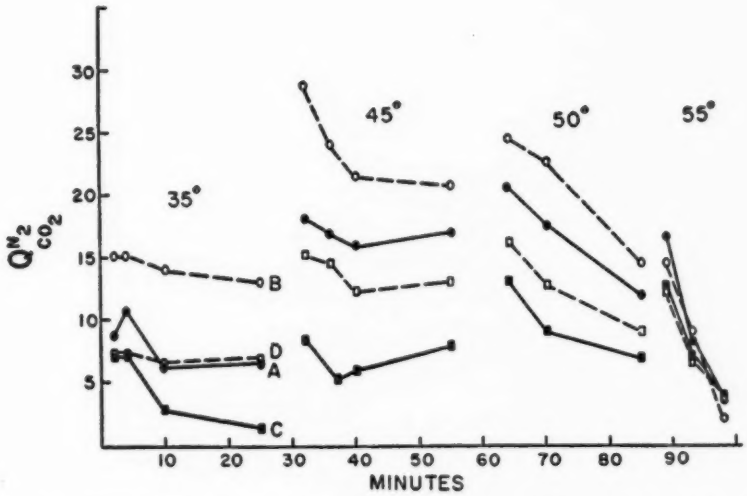
The *in vitro* effects of temperature, exogenous insulin, and prior exposure to stress upon the extent of the testosterone-induced inhibition of glycolysis is further illustrated in table 6 which summarizes data obtained from two S91 melanomas exposed to 20° C. for 17 hours and to 35° C. for 67



TEXT-FIGURE 10.—$Q^{N_2}_{CO_2}$ time curves (at 20° C.) of slices (200 mg. wet weight per vessel) of a 35° S91 melanoma (19 hours' stress) in KRBC (0.5% glucose, 6.1 ml.) plus 2.2 *gamma* ionic zinc per ml. A=control (minus insulin); B=plus 19 *gamma* (0.5 unit) low-zinc insulin per ml.; C=plus 65 *gamma* testosterone per ml.; D=plus testosterone and insulin.

hours, respectively. Incubation was in KRBC plus 0.54 percent glucose (5.8 ml. total vol.), zinc insulin [20 *gamma* (0.5 unit) per ml.], and testosterone (68 *gamma* per ml.). It can be seen that the greatest average inhibition occurred in the 35° tumor and that in each tumor there was a tendency for exogenous insulin to lower inhibition. It is clear that increasing the temperature of incubation reversibly lessened the testosterone-induced inhibition of glycolysis, particularly in the 20° tumor. There was a progressive lowering of $Q^{N_2}_{CO_2}$ at 45° C. in the presence of testosterone which seemed to be of an irreversible nature. The data of

this and other experiments indicated that the thermal maximum (temperature above which glycolysis becomes irreversibly inactivated) for S91 melanoma may be lowered by testosterone. It should be noted, however, that below the thermal maximum (*e.g.*, at 20° C.) $Q^{N_2}_{CO_2}$, after equilibration, became steady in the presence of testosterone (*e.g.*, as long as 1,100 minutes of incubation).



TEXT-FIGURE 11.—$Q^{N_2}_{CO_2}$ time curves of same tissue as in text-figure 10, but following transfer to indicated higher temperatures. A=control; B=plus insulin; C=plus testosterone; D=plus insulin and testosterone. Zero on time scale is approximately 240 minutes from start of incubation at 20° C. (text-fig. 10).

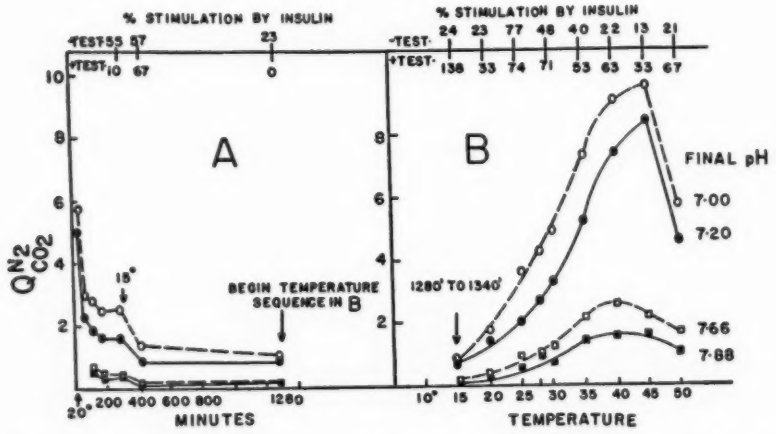
TABLE 6.—*Effect of temperature and zinc insulin on percent inhibition of* $Q^{N_2}_{CO_2}$ *by testosterone*

Incubation temperature sequence	Percent inhibition by testosterone*			
	Tumor from mouse exposed to 20° C. for 17 hours		Tumor from mouse exposed to 35° C. for 67 hours	
	−Insulin	+Insulin	−Insulin	+Insulin
20° (1st cycle)	38	28	46	42
35° (1st cycle)	28	21	46	43
20° (2nd cycle)	54	33	85	66
35° (2nd cycle)	—	—	55	43
45° (1st cycle)	34	23	(1st 31 min. only = 54; total 61-min. period = 67)	(1st 31 min. only = 39; total 61-min. period = 50)

*Averages for total period of incubation at each temperature.

The effects of exogenous insulin and testosterone over a wide range of incubation temperatures are illustrated in text-figure 12. In this experiment, four S91 melanomas, from male DBA mice previously stressed at 35° C. for 24 hours, were sliced and pooled. One aliquot of the pooled slices was washed (at 0 to 5° C.) in three successive 20-ml. volumes of KRBC plus 0.625 percent glucose. Both washed and unwashed slices were run. Since both lots of tissue gave essentially the same results, and since in most other instances unwashed slices have been used, only the data from the unwashed tissue are presented in the text-figure. The data of this and other experiments show that the phenomena observed were due to factors that were not released by repeated washing of the tissue. Text-figure 12A shows the curves obtained during initial equilibration for 275 minutes at 20° C., followed by incubation for 915 minutes at 15° C. Four conditions are compared, *i.e.*, plus or minus exogenous insulin and plus or minus testosterone. The total elapsed time of the *in vitro* experimentation, including incubation at all temperatures, was 1,744 minutes. A number of other long-term incubations have shown that in the presence of the levels of glucose and bicarbonate employed here, *in vitro* glycolysis of S91 melanoma slices is remarkably stable, especially in the temperature range 15 to 20° C.

For the first 55 minutes at 15° C. (following initial equilibration at 20° C.) the average percent stimulation by insulin was 57 percent. This fell to 23 percent during the next 860 minutes *solely as a result of further*



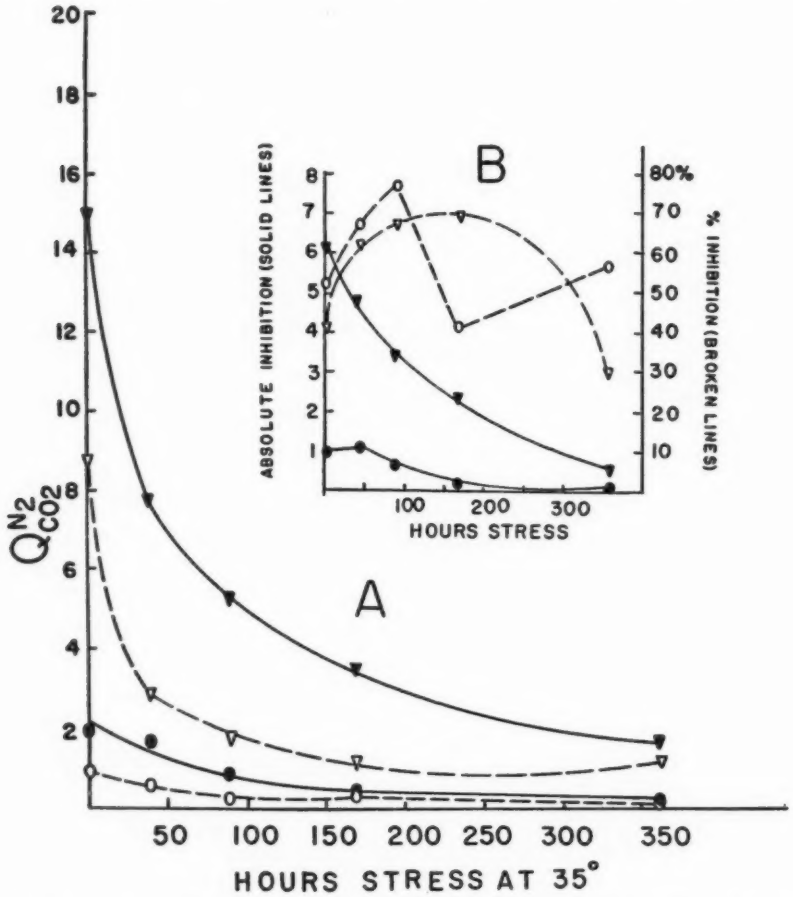
TEXT-FIGURE 12.—A=$Q^{N_2}_{CO_2}$ time curves (20 to 15° C.) of pooled slices (200 mg. wet weight per vessel) of four 35° S91 melanomas (24 hours' exposure) in KRBC plus 0.55 percent glucose (5.7 ml.); ●—control (minus insulin); ○—plus 14 *gamma* (0.36 unit) zinc insulin per ml.; ■—plus 70 *gamma* testosterone per ml.; □—plus testosterone and insulin. B=$Q^{N_2}_{CO_2}$ values at approximate glycolytic equilibrium following transfer to indicated successive temperatures of incubation (°C.). Percent stimulation by insulin, in the presence and absence of testosterone, is indicated for both A and B on the horizontal axis at top of graph.

decline in the $Q_{CO_2}^{N_2}$ with insulin (text-fig. 12A). It is noteworthy that in the presence of testosterone the $Q_{CO_2}^{N_2}$ values were very low (0.1 to 0.18) but remained essentially constant throughout the 975 minutes of incubation at 15° C. The curves in text-figure 12B are based on the approximate equilibrium $Q_{CO_2}^{N_2}$ values for each condition at successively higher temperatures of incubation, beginning with the last 60 minutes at 15° C. (1,280 to 1,340 minutes from zero time). $Q_{CO_2}^{N_2}$ increased with rising temperature up to 45° C., although in the presence of testosterone the *maximum* $Q_{CO_2}^{N_2}$ was at 40° C. instead of at 45° C. It should be noted that in some experiments the maximum $Q_{CO_2}^{N_2}$ values have been obtained at temperatures above 45° C. (*ca.* 50° C.), possibly as the result of variations in concentration of endogenous inhibitors, previous history of incubation, etc. In the absence of added testosterone, raising the temperature from 15 to 20° C. approximately doubled the $Q_{CO_2}^{N_2}$ (*ca.* 115% increase plus or minus insulin) without significantly altering the percentage stimulation by exogenous insulin (24 and 23% stimulation at respective temperatures). However, with a further increase in temperature from 20 to 35° C., the average increase in $Q_{CO_2}^{N_2}$ was only 38 percent in the absence of insulin and 46 percent in the presence of insulin. This resulted in an increase in the percent insulin effect from 23 percent at 20° C. to 40 percent at 35° C. Percentage insulin effect was maximal at 25° C. (77% stimulation). At 40° C. and above the percentage insulin effect declined sharply. Comparable values for the testosterone series are also presented in text-figure 12B. Testosterone, as usual, tended to increase the percentage insulin effect.

The analysis of many experiments involving sequential changes in temperature of incubation has shown that an increase in temperature tends to increase insulin action relatively more than it does anti-insulin action. *This is true whether the endogenous inhibitor mechanism alone is involved or whether this system is further potentiated by added testosterone.* Again it should be emphasized that the optimum temperature for demonstrating the stimulation of glycolysis by exogenous insulin will vary among different tumors, depending on the extent of prior stress, concentration of glucose in the medium, phases of the time-course curves of $Q_{CO_2}^{N_2}$ compared, etc. The data suggest that the level at which the glucose metabolism of a tumor will be poised can be strongly dependent on the endocrine balance of the host (here modified experimentally by degree of exposure to temperature stress) and the actual temperature of the tissues. The latter will, of course, usually be relatively constant except in special cases (animals with very large tumors, poorly vascularized areas of large exposed tumors, etc.). It is particularly significant in this connection that the effect of temperature on glycolytic inhibition induced by exogenous testosterone is similar to the effect of temperature on the stress-modifiable endogenous inhibitor mechanism.

Duration of stress and sensitivity to testosterone.—Because both prior temperature stress and the addition of testosterone lowered the *in vitro* $Q_{CO_2}^{N_2}$, it was of interest to know to what extent these two inhibition-

promoting factors were additive. Text-figure 13 illustrates the relation between length of exposure of the host-tumor system to 35° C., and subsequent *in vitro* sensitivity of the tumor to testosterone (*ca.* 70 *gamma* per ml.). It can be seen that *absolute* inhibition of $Q_{CO_2}^{N_2}$ by testosterone was greatest in the tumors of mice that had not been exposed to 35° C. and became progressively less as the exposure period was prolonged (text-



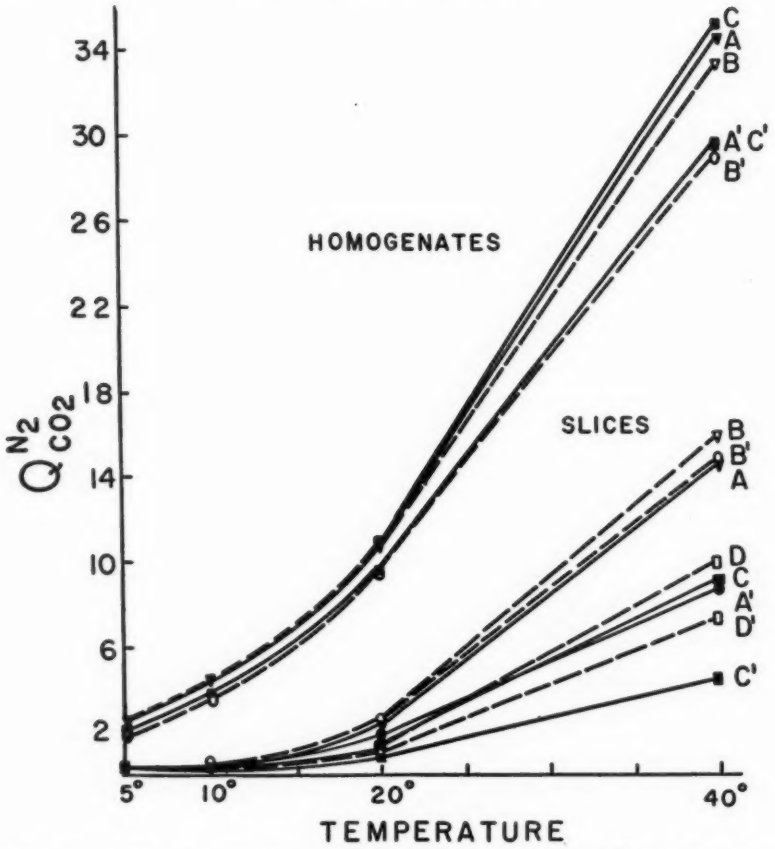
TEXT-FIGURE 13.—The relation between length of exposure of the host S91 melanoma system to temperature stress (at 35° C.) and response ($Q_{CO_2}^{N_2}$ of slices) to *in vitro* addition of testosterone. All incubations were in KRBC with about 0.6 percent glucose and 70 *gamma* testosterone per ml. A (main curves) = $Q_{CO_2}^{N_2}$ *versus* hours of stress; ●, ○—incubated at 20° C.; ▼, ▽—incubated at 40° C. (closed figures = minus testosterone; open figures = plus testosterone). B (inset, *upper right*) = ●▼—absolute inhibition of $Q_{CO_2}^{N_2}$ by testosterone (circles, at 20° C.; triangles, at 40° C.); ○▽—percent inhibition in $Q_{CO_2}^{N_2}$ by testosterone (circles, at 20° C.; triangles, at 40° C.).

fig. 13, solid line of inset). On the other hand, *percentage* inhibition (broken line of inset) of $Q_{CO_2}^{N_2}$ by testosterone increased from zero exposure to a maximum at 90 to 168 hours of exposure and then fell off. In line with previous data the absolute value curves were much flatter at 20° C. Other experiments showed that one third the concentration of testosterone employed here, *i.e.*, only 23 *gamma* per ml., produced as much inhibition as 69 *gamma* per ml. Furthermore, they have shown that the effects of a given amount of exposure to stress cannot be replaced by merely increasing the concentration of testosterone. In other words *in vitro addition of testosterone cannot completely replace the effects of prior exposure to stress.* However, sufficient prolongation of temperature stress can result in a glycolytic inhibition such that subsequent *in vitro* exposure to testosterone will produce little further inhibition. These results are consistent with the view that the endogenous stress-modifiable inhibitor mechanism involves a factor that is not equivalent to testosterone (*i.e.*, presumably nonsteroidal) but is, however, strongly potentiated either by endogenous steroid(s) or exogenous testosterone. Conceivably the concentration of endogenous steroid(s) becomes saturating after very prolonged stress. The apparently nonsteroidal factor may well be the pituitary anti-insulin factor reported by others (*2*, *10–12*), but not growth hormone, which we have tested.

Tumor Slices versus *Homogenates*

Previous work (*13*) had shown that cell-free homogenates of S91 melanoma would yield an active glycolysis if fortified with certain cofactors (diphosphopyridine nucleotide, adenosinetriphosphate, niacinamide, PO_4, Mg) and hexose diphosphate. It was of interest to determine whether such fortified subcellular systems, prepared from melanomas of stressed and "unstressed" mice, would show hormonal and temperature sensitivities comparable to slices of the same tumors. Tumors were sliced as in the preceding experiments and aliquots of the thoroughly mixed tissue were then homogenized at 0 to 5° C. (*ca.* 10% by weight of tissue in KRBC) in a Potter-Elvehjem-type homogenizer until 95 to 98 percent of the cells were broken up. From 5 to 10 minutes were usually required for homogenization. In some experiments, the pooled slices were washed 3 times or more before preparing the homogenate. While this procedure had no appreciable effect on the $Q_{CO_2}^{N_2}$ (based on original weight of tissue) of the slices, it did remove broken cell material which had an appreciable $Q_{CO_2}^{N_2}$ in the cofactor-fortified solution. Since a number of experiments gave essentially the same results, data from a typical experiment will be presented.

Text-figure 14 shows curves obtained by plotting approximately steady rate $Q_{CO_2}^{N_2}$ values of S91 melanoma slices at four successive temperatures of incubation. In one case (A, B, C, D, text-fig. 14) the tumors were from mice maintained at 25° C., and in the other (A′, B′, C′, D′) from mice exposed to 35° C. for 48 hours. The upper group of curves (corresponding lettering) shows $Q_{CO_2}^{N_2}$ values for homogenates of the respective slice



TEXT-FIGURE 14.—Comparative anaerobic glycolytic activities ($Q^{N_2}_{CO_2}$) of slices (*lower group of curves*) of 25° S91 melanoma (A, B, C, D) *versus* 35° melanoma (A′, B′, C′, D′); stressed 48 hours. Approximate equilibrium values for corresponding time periods for homogenates (*upper group of curves*) derived from respective slice preparations have been plotted. SLICES: A=control (minus insulin); B=plus 14 *gamma* (0.36 unit) zinc insulin per ml.; C=plus 70 *gamma* testosterone per ml.; D=plus insulin and testosterone. HOMOGENATES: A=control; B=plus 35 *gamma* (0.91 unit) zinc insulin per ml.; C=plus 182 *gamma* testosterone per ml. (A′, B′, C′ are corresponding 35° tumor curves). Slices (200 mg. wet weight per vessel) were run in KRBC plus 0.55 percent glucose (5.7 ml.); homogenates (1 ml. of 10% tissue concentrations) were prepared in modified KRBC (see text) and run at 0.43 percent glucose concentration (2.2 ml.).

preparations. These homogenates did not have a significant $Q^{N_2}_{CO_2}$ until the cofactors were tipped in. Zinc insulin and testosterone were in all cases added to the vessels before incubating them at 5° C.

The homogenate responses (upper curves) were in marked contrast to those obtained with the slices. *There were no significant effects of insulin or testosterone, and the activities of the 35° tumor homogenate were only*

slightly below those of the 25° homogenate. Furthermore, there were marked differences between slices and homogenates with respect to the responses of $Q_{CO_2}^{N_2}$ to temperature change. In general, it can be said that there were no significant alterations in the temperature coefficients of the homogenates due to prior exposure to stress or to the presence of exogenous insulin and/or testosterone (table 7). The temperature coefficients for the homogenates, except in the 5 to 10° C. temperature range, were much lower than in the slices, and the Q values were higher.

It is clear from the results of a number of experiments that, in cofactor-fortified S91 melanoma homogenates of the type just described, there is a complete by-passing of the insulin : anti-insulin glycolysis-regulating mechanism which operates in intact cells. It should be noted that no inference is intended that such mechanisms cannot operate at the subcellular level in other types of preparations. Studies along this line are actively under way in this laboratory (Hochstein, unpublished data).[6]

Nonmalignant Tissues and Temperature Stress

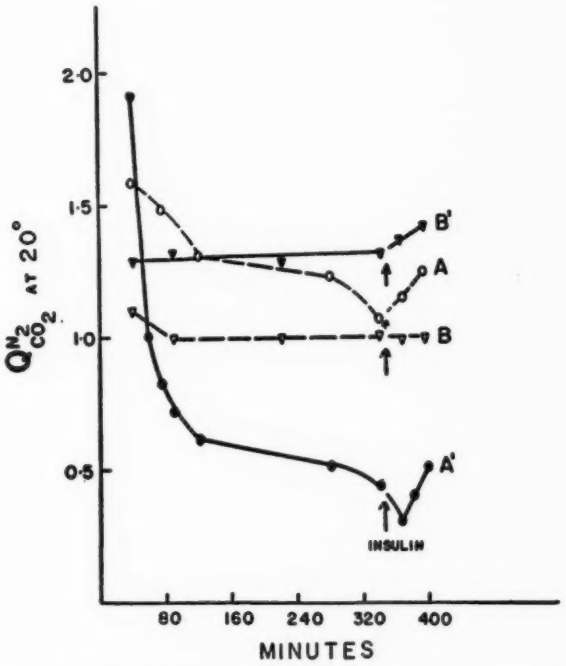
Previous studies (*1*) had shown that exposure of either normal or S91 melanoma-bearing mice to stressing temperatures did not produce significant changes in $Q_{CO_2}^{N_2}$ of the brain, and insulin had no stimulatory effect. However, more recently (*14*) it has been possible to demonstrate positive effects of insulin in brain slices under aerobic conditions, although, as might be expected from the normal behavior of the temperature-treated animals, no clear-cut abnormality occurred in the metabolism of slices not treated with insulin. The *in vitro* data so far indicate [(*14*) and unpublished] that while *in vitro* brain metabolism appears to be under the control of a regulatory mechanism highly sensitive to temperature, in some respects resembling that of the S91 melanoma, the equilibrium between insulin and anti-insulin factors is much more difficult to alter either by imposition of temperature stress on the whole animal or by addition of insulin *in vitro*. In the present investigation, measurements have been made of the effect of prolonged exposure of S91 melanoma-bearing mice to 35° C. on the $Q_{CO_2}^{N_2}$ of both tumor and kidney tissue. The data show that glucose metabolism of the kidneys is under control of a regulatory mechanism which responds *in vitro* to changes in incubation temperature and to testosterone in a manner resembling S91 melanoma. However, as in brain, but unlike tumor, the system appears to be strongly resistant to unbalancing as a result of stress.

Text-figure 15 shows the $Q_{CO_2}^{N_2}$ time curves at 20° C. for an S91 melanoma (curve A) and the kidneys (curve B) of one strain DBA mouse maintained at 25° C. Curves A′ and B′ show the $Q_{CO_2}^{N_2}$ time curves for the tumor and kidneys of another mouse of the same transplant generation exposed for 16 days to 35° C. Zinc insulin was tipped as indicated by the arrows. The initial glucose concentration was 0.6 percent (5.2 ml.). After 396

[6] The following abstract has appeared since this paper went to press: Insulin-reversible, stress-induced inhibition of cell-free glycolysis in S91 mouse melanoma homogenates. Paul Hochstein, Mark Woods, and Dean Burk. Fed. Proc. 14: 227, 1955.

TABLE 7.—*Effect of changes in incubation temperatures on* $Q_{CO_2}^{N_2}$ *of S91 melanoma slices and homogenates*

Temperature change (°C.)	Percent increase in $Q_{CO_2}^{N_2}$ following temperature change							Percent increase in presence of testosterone *minus* percent increase without testosterone		
	25° Tumor slices				25° Homogenate			25° Slices		25° Homog.
	−Insulin	+Insulin	+Testosterone		−Insulin	+Insulin	+Testosterone	−Insulin	+Insulin	−Insulin
			−Insulin	+Insulin			−Insulin			
5°→10°	38	97	50	69	80	112	76	12	−28	−4
10°→20°	386	314	218	156	141	146	152	−168	−158	+11
20°→40°	494	549	531	625	232	210	220	37	76	−12
	35° Tumor slices				35° Homogenate			35° Slices		35° Homog.
5°→10°	30	103	−13	−10	113	84	93	−43	−113	−20
10°→20°	283	315	200	163	156	180	146	−83	−152	−10
20°→40°	368	487	487	700	211	204	211	119	213	0



TEXT-FIGURE 15.—$Q^{N_2}_{CO_2}$ time curves for slices of S91 melanoma (circles), and the kidneys (triangles) of the same animal. A=25° melanoma; B=25° kidneys; A′=35° melanoma (16 days' exposure to 35°); B′=35° kidneys. In KRBC with 0.6 percent glucose (5.2 ml.). Zinc insulin was tipped in as indicated to give a final concentration of 15 *gamma* (0.39 unit) per ml. Tissue concentrations (wet weight) per vessel were 350 mg. for tumors and 200 mg. for kidneys.

minutes of incubation at 20° C., the tissue was transferred to 40° C. The average $Q^{N_2}_{CO_2}$ values for the first 30 minutes of incubation at 40° C. were for the 25 and 35° tumors, respectively, 10.3 and 3.1, and for the corresponding kidneys, 5.1 and 6.1. These results, and those of other experiments, show that prolonged exposure of tumor-bearing animals to temperature stress does not lower the anaerobic glycolytic capacity of kidney, and exogenous insulin has either little or no effect.

S91A Amelanotic Melanoma

Degree of Cell Variation

It has long been known that, in keeping with the situation in certain human melanomas, there is a tendency for the transplantable S91 melanoma to develop cellular lines with wide variations in melanin content. Algire (*5*) first succeeded in establishing essentially amelanotic strains of the S91 tumor, and Hesselbach (*15*, *16*) has presented evidence from extensive transplantation experiments that maintenance of the amelanotic

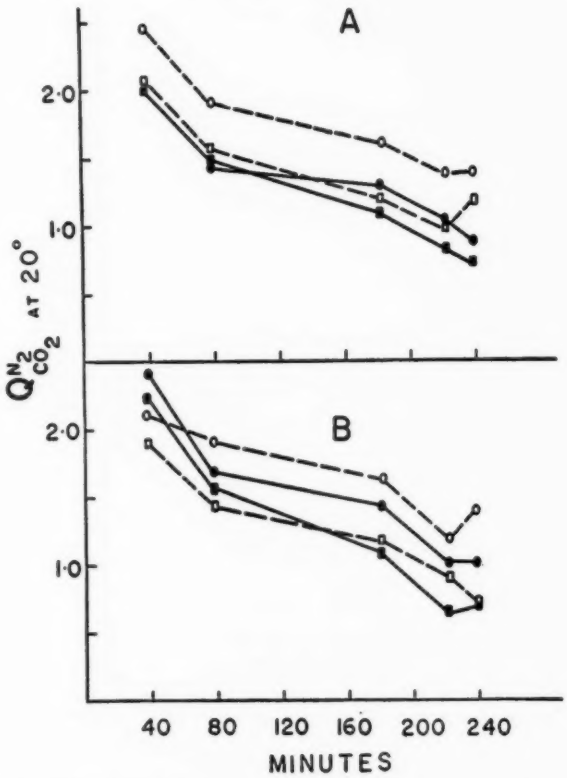
condition does not depend upon the capacity of the host to form melanin. She has suggested, moreover, that the growth rates of melanotic and amelanotic cellular derivatives may not be the same in different host strains (DBA *versus* C), thus accounting for the reported influence of host strain on melanization tendency [(*15*) p. 207]. Indeed, results secured in this laboratory (*17*) show that the S91 melanoma grows faster in DBA than does the derived amelanotic S91A tumor. On the other hand, it has been our observation (unpublished) that in the "foreign" C strain of mice the S91A tumors grow faster than the S91 tumors. Previous studies (*13*, *18–20*) led to the view that melanization, or lack of melanization, in these mouse tumors is controlled by enzyme activities centered in the mitochondria. Individual S91 tumor cells have been found which contained irregularly distributed melanized and nonmelanized mitochondria. This led to the suggestion [(*18*) p. 322] that ". . . in some areas [of the tumor] these two types of granules became separated, forming completely melanotic or completely amelanotic cells. The presence in the same tumor of completely melanotic and completely amelanotic cells suggests the possibility of cytoplasmic segregation similar to that observed in plant cells." This view has been further emphasized by Hesselbach (*16*). DuBuy *et al.* [(*13*) p. 350] have reported that ". . . the predominantly melanized cytoplasmic particulate fractions of the S91 melanoma possessed glycolytic activities that were in general comparable to the nonmelanized mitochondrial and subvisible particle fractions of hepatoma and liver." It was therefore of particular interest in the present study to compare the patterns of hormonal regulation of glycolysis in both the melanized S91 and the derived amelanotic S91A tumor. It should be emphasized that while the accumulated evidence indicates that the appearance of amelanotic cells represents the attainment of a further degree of anaplasia within the malignant cell population, the differences between the two types of tumors are not limited to melanin content alone. Differences in growth rate, tendency to metastasize, and host specificity have all been found (*5*, *21*). The presence, or relative absence, of melanization may therefore be considered as only one expression of variation within the population of tumor cells, not necessarily related to changes in hormone sensitivity.

Effect of Stress, Testosterone, and Insulin on $Q_{CO_2}^{N_2}$

Repeated experiments have shown that exposure of mice bearing S91A amelanotic tumors to a constant temperature of 35° C. elicits much less lowering of glycolysis than in comparably treated S91 melanomas. *In vitro* sensitivity to insulin and testosterone is likewise much less marked in the amelanotic tumors. Text-figure 16 shows the $Q_{CO_2}^{N_2}$ time curves at 20° C. for two S91A amelanotic melanomas (A=25° tumor; B=35° tumor, 92 hours' stress) grown in DBA mice. The over-all activities were not markedly different between the two tumors. Thus if the average $Q_{CO_2}^{N_2}$ values of the 25° tumor are divided by the corresponding values for the 35° tumor, the average for all treatments at 20° C. is 1.01 (range 0.91 to 1.03), and at 40° C. is 0.96 (range 0.94 to 0.97). However, the testosterone

curves for the 35° tumor (text-fig. 16B) are definitely below the control, whereas in the 25° tumor (text-fig. 16A) the rate with testosterone plus insulin is more nearly equal to the control. Following transfer to 40° C. the approximate equilibrium $Q_{CO_2}^{N_2}$, values were for the 25° *versus* the 35° tumors, controls 8.1 *versus* 8.4, plus insulin 8.8 *versus* 8.9, plus testosterone 6.4 *versus* 6.2, and testosterone plus insulin 7.1 *versus* 6.3. Final *p*H values were in the range 7.28 to 7.43. These data clearly show a much lower degree of response to prior temperature stress than in comparably treated S91 melanomas.

Table 8 presents data from another experiment with S91A amelanotic melanoma that represents the upper limits of observed responses to temperature stress. Reagent and tissue concentrations were the same as in the experiment shown in text-figure 16. While larger insulin and testosterone effects were obtained than in the preceding experiment, they were



TEXT-FIGURE 16.—$Q_{CO_2}^{N_2}$ time curves (at 20° C.) of S91A amelanotic melanoma slices (200 mg. per vessel) in KRBC with 0.55 percent glucose (5.7 ml.). A=25° tumor; ●—control (minus insulin); ○—plus 14 *gamma* (0.36 unit) zinc insulin per ml.; ■—plus 70 *gamma* testosterone per ml.; □—plus insulin and testosterone. B=35° tumor (92 hours' exposure to 35° C.). All symbols correspond to those in A.

TABLE 8.—*Anaerobic glycolysis of S91A amelanotic melanoma slices from temperature-stressed and "unstressed" DBA mice*

Temperature and time (*minutes*)	25° Tumor					35° Tumor (*68 hours' exposure*)				
	$Q_{CO_2}^{N_2}$			Percent effect		$Q_{CO_2}^{N_2}$			Percent effect	
	−Insulin	+Insulin	+Testosterone	Insulin	Testosterone	−Insulin	+Insulin	+Testosterone	Insulin	Testosterone
20°										
38–48	1. 73	1. 27	0. 66	−27	−62	1. 29	1. 30	0. 75	+1	−42
68	1. 59	1. 46	0. 85	−8	−47	1. 27	1. 44	0. 58	+13	−54
128	0. 86	0. 70	0. 51	−19	−41	0. 47	0. 78	0. 43	+66	−9
To 40° at 165′										
173–183	8. 05	8. 13	3. 42	+1	−58	3. 08	7. 10	2. 69	+131	−13
193	6. 53	6. 25	2. 95	−4	−55	5. 31	6. 11	2. 21	+15	−58
218	6. 39	6. 47	3. 51	+1	−45	5. 25	6. 28	2. 61	+20	−50
238	5. 29	5. 37	3. 46	+2	−35	5. 05	5. 68	1. 62	+12	−68
258	6. 12	5. 80	3. 16	−5	−48	5. 13	5. 88	1. 82	+15	−65
Final *p*H	*7. 41*	*7. 45*	*7. 52*	.....	.....	*7. 46*	*7. 40*	*7. 60*		

still below the S91 melanoma level for comparably treated tumors. It can be seen (table 8) that zinc insulin strongly counteracted the inhibition of glycolysis resulting from prior exposure of host and tumor to 35° C., although the average percent of insulin effect in the 35° tumor at 40° C. after the first 10 minutes of incubation was only 15 percent. This is well below the average of S91 melanomas measured under comparable conditions. As in S91 melanoma, prior stress (35° C. for 68 hours) resulted in an increase in sensitivity to exogenous testosterone (table 8, incubation at 40° C.), although the effect was of a low order.

It can be seen from column 5 of table 8 that at 20° C. insulin actually inhibited glycolysis in the 25° tumor, although following transfer to 40° C. this effect was not apparent. Negative effects of insulin have been observed under certain conditions in other tumors (*e.g.*, Harding-Passey melanoma, human melanoma) and do not appear to be the result of experimental variability. In most cases, the inhibitory effects of insulin preparations must be more than compensated by the stimulatory action of the hormone.

The average $Q^{N_2}_{CO_2}$ values for S91A amelanotic melanomas have been significantly lower than in comparable S91 melanomas. This difference is best illustrated by comparing the *in vitro* $Q^{N_2}_{CO_2}$ of tumor slices from animals not previously exposed to temperature stress and incubated at 40° C. in KRBC with insulin plus 0.6 percent glucose. In 6 such S91A tumors, the average observed *maximum* $Q^{N_2}_{CO_2}$ was 11.47 (range 8.13 to 17.50), and the average value at approximate glycolytic equilibrium was 8.13 (range 5.60 to 10.10). On the other hand, in 6 comparable, randomly selected experiments with S91 tumors the average observed *maximum* $Q^{N_2}_{CO_2}$ was 20.10 (range 19.13 to 25.93), and the average approximate equilibrium value was 18.11 (range 15.11 to 25.62). The average percentage effect of exogenous insulin was also much lower in S91A tumors from stressed mice than in comparable S91 tumors. In S91 melanoma the average percent insulin effect in tumors from mice stressed at 35° C. was about 50 percent (34 to 98%). The average for 7 comparable S91A tumors was 14.9 percent (8 to 28%). Comparable values for "unstressed" (20 to 25° C.) mice were 7.0 percent (2 to 14%) in S91 melanoma, and 4.7 percent (−4 to 15%) in S91A amelanotic melanoma.

The accumulated data from all sources make it evident that the S91A amelanotic tumors are more anaplastic than the predominantly melanized parental S91-type tumors. Among the manifestations of this progression in anaplasia is an increased resistance to the anti-insulin hormonal mechanism which plays such an important part in regulating glucose metabolism of the parent tumor. The lower basic $Q^{N_2}_{CO_2}$ of the S91A tumor is another difference which may or may not be directly related to the change in hormone sensitivity. The striking parallel between stress-induced inhibition of glycolysis in S91 melanoma and inhibition in tumor growth has already been reported (*1*). The data suggest, but do not prove, that hormonal inhibition of glucose utilization on the one hand, and inhibition in tumor growth on the other, are directly related. On this basis

it might be predicted from the metabolic data obtained with S91A tumors that temperature stressing would produce partial, but not complete, inhibition of growth in this derived tumor. The accompanying report by one of the authors (*17*) shows that this is indeed the case.

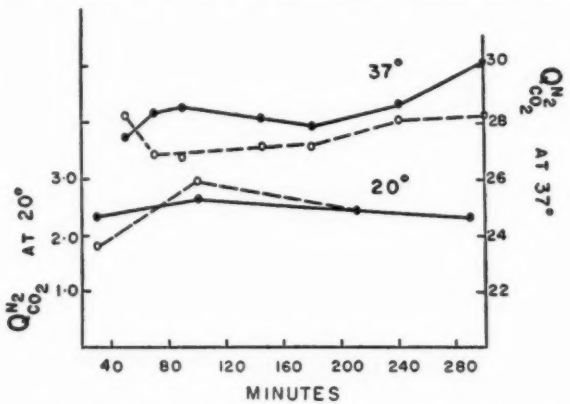
Krebs-2 Carcinoma

The Krebs-2 carcinoma (*6*) has been widely used in experimental studies of cancer. The work of Hauschka and associates (*22*) has shown that the usual cell populations of this tumor, grown in ascites form, are mixtures of cell races differing in a number of properties. We have used gross transfers of the ascites form (*ca.* 0.2 ml. of undiluted extract) into the peritoneal cavity of mice to produce typical ascites tumors, or smaller doses (0.1 ml.) introduced intramuscularly to produce solid tumors. Since this tumor is characterized by a high $Q^{N_2}_{CO_2}$ (*23*) and has a low degree of host-specificity (*22*), it was of special interest to determine its sensitivity to the insulin : anti-insulin mechanisms effective in regulating the metabolism of the S91 melanoma. Both ascites and solid forms of the Krebs-2 tumor were studied in DBA and C mice. There were no observed differences due to host strain.

Ascites Tumor

$Q^{N_2}_{CO_2}$, *time curve.*—The types of curves obtained from the time course of glycolysis in Krebs-2 ascites tumors (text-fig. 17) were very different from those which characterized the S91 melanoma (see text-figs. 2, 3, and 4). The glycolytic rates of Krebs 2 instead of falling off with time remained nearly level or increased slightly. Insulin gave no significant stimulation.

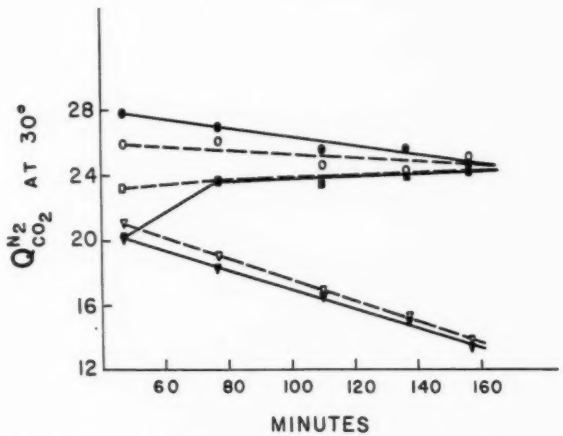
Effect of stress and insulin.—Metabolic examination of Krebs-2 ascites tumors grown in mice exposed to stressing temperatures (35 to 40° C.)



TEXT-FIGURE 17.—$Q^{N_2}_{CO_2}$, time curves for 23° Krebs-2 ascites tumor (1 ml. of 1 : 10 dilution in KRBC per vessel) with 0.57 percent glucose (3.3 ml. total volume) incubated at 20° C. (*lower curves*) or at 37° C. (*upper curves*); ●—control (minus insulin); ○—plus 35 *gamma* (0.9 unit) zinc insulin per ml.

failed to reveal any evidence of a stress-induced, insulin-reversible lowering of glycolysis. For example, an 8-day-old ascites tumor (1:10 dilution of ascitic extract in KRBC) from a male strain C mouse which had been exposed to 39.5° C. for 26 hours had a $Q_{CO_2}^{N_2}$ (at 38° C. with 0.5% glucose) of 45 in the absence of exogenous insulin, and 42 in the presence of 78 *gamma* (2 units) of zinc insulin per ml. Corresponding values for a comparable control tumor from a mouse maintained at 25° C. were 38.5 and 37.1, respectively.[7] Repeated attempts to demonstrate stimulation of glycolysis by insulin (zinc or low-zinc types) in Krebs-2 ascites tumors have failed to show significant effects (average effect in 10 experiments −0.6%, range −8 to 9%). These results suggested that the Krebs-2 ascites populations in our tumor cultures were highly resistant to the insulin-reversible glycolytic inhibition which is accentuated by temperature stress. It should be noted that there was a much higher mortality at 35 or 40° C. among mice with Krebs-2 ascites tumors than in comparable mice held at 25° C.

Effect of steroids.—The insensitivity of Krebs-2 ascites tumors to temperature stress and insulin parallels their lack of sensitivity to *in vitro* testosterone. While diethylstilbestrol causes a progressive inhibition of glycolysis, the effect is not reversed by insulin (text-fig. 18). The progressive inhibition caused by diethylstilbestrol, which is not acting over an insulin-reversible mechanism, appears to be of a nonspecific character. Similar effects of this estrogen have been observed in S91 melanoma



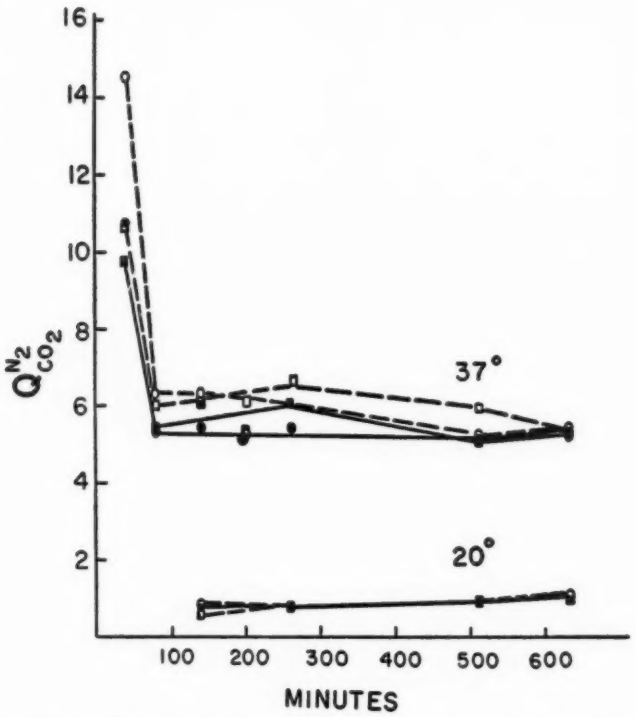
TEXT-FIGURE 18.—$Q_{CO_2}^{N_2}$ time curves of 25° Krebs-2 ascites tumor (2 ml. of 1:10 dilution of ascitic extract in KRBC) incubated at 30° C. with 0.43 percent glucose (4.2 ml. total volume); ●—control (minus insulin); ○—plus 19 *gamma* (0.49 unit) zinc insulin per ml.; ■—plus 190 *gamma* testosterone per ml.; □—plus testosterone and insulin; ▼—plus 190 *gamma* diethylstilbestrol per ml.; ▽—plus stilbestrol and insulin.

[7] While $Q_{CO_2}^{N_2}$ values for Krebs-2 ascites tumors in KRBC have usually fallen within the range 35 to 50, under special conditions values from 60 to well over 100 have been obtained.

cofactor-fortified homogenates where testosterone and insulin had no effect (see previous section). Wight and Burk (*24*) have reported that diethylstilbestrol markedly inhibited the succinoxidase activity of S91 and Harding-Passey subcellular particulates (mitochondria). The small inhibition caused by testosterone in the Krebs-2 ascites tumor (text-fig. 18) may have been of a similar nonspecific character, since addition of insulin gave no significant reversal.

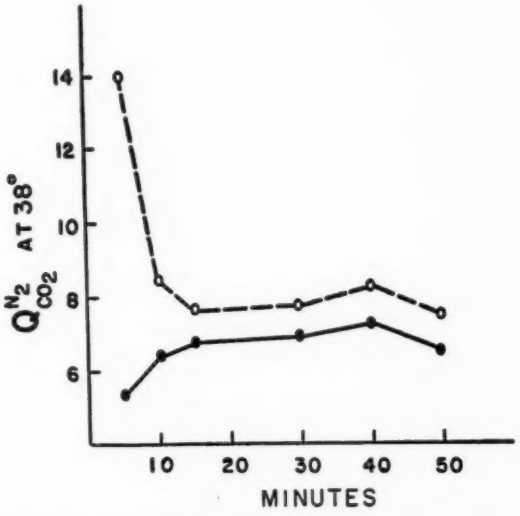
Solid Tumor

Some solid Krebs-2 tumors responded significantly to insulin, others behaved like the ascites forms. The average $Q_{CO_2}^{N_2}$ of the solid Krebs-2 tumors was considerably lower than in the ascites forms. Thus in 6 solid tumors the average without insulin was 7.22 (5.30 to 10.30), and with insulin 7.55 (5.98 to 10.70). It can be seen from text-figure 19 that prior temperature stress (35° C. for 20 hours) and *in vitro* zinc insulin had little effect on $Q_{CO_2}^{N_2}$, either at 20 or 37° C, incubation. Text-figure 20 illus-



TEXT-FIGURE 19.—$Q_{CO_2}^{N_2}$ time curves for slices (50 mg. wet weight per vessel) of solid Krebs-2 tumors in KRBC with 0.57 percent glucose (2.2 ml. total volume) at incubation temperatures at 20° C. (*lower curves*) or 37° C. (*upper curves*); ●—25° tumor control (minus insulin); ○—25° tumor plus 35 *gamma* (0.9 unit) zinc insulin per ml.; ■—35° tumor (20 hours' stress) control; □—35° tumor plus insulin.

trates the $Q^{N_2}_{CO_2}$ time curves for a solid Krebs-2 tumor in which the largest observed response to insulin in this tumor was obtained. At approximate glycolytic equilibrium the percentage stimulation by insulin was approximately 14 percent, although it was much larger initially. While the medium employed in this case was Hanks-Simms' [as used previously (*1*)] and the insulin concentration was higher than usual (78 *gamma* or 2 units per ml.), the insulin effect was apparently not due to these factors, since repetitions, under the same conditions, with Krebs-2 ascites tumors failed to yield significant insulin effects.



TEXT-FIGURE 20.—$Q^{N_2}_{CO_2}$ time curves of slices (100 mg. wet weight per vessel) of solid 25° Krebs-2 tumor in Hanks-Simms' medium with 0.5 percent glucose (1.25 ml. total volume); ●—minus insulin; ○—plus 78 *gamma* (2 units) zinc insulin per ml.

Harding-Passey Melanoma

With the exception of the Cloudman S91 tumor, the Harding-Passey (H-P) melanoma was the only mouse melanoma available for experimentation. In general the H-P melanoma is less anaplastic than the S91 tumor with respect to cytomorphic characteristics (*18*), metastatic capacity (*21*), and growth rate (*21*). However, unlike the S91 tumors, our cultures have grown almost equally well in various host genotypes (C, DBA, C3H). We have examined the metabolic responses of H-P tumors from both C and DBA mice and have noted no significant differences; therefore the results from tumors grown in both host strains will be combined.

Magnitude of $Q^{N_2}_{CO_2}$

The $Q^{N_2}_{CO_2}$ of H-P melanomas is much lower than that of any other mouse tumor employed in this investigation. For example, the average

equilibrium value for 5 H-P melanomas in KRBC (0.6% glucose at 34 to 40° C.) was 2.03 (1.19 to 2.66). In Warburg-Okamoto solution plus 0.2 percent glucose [as used in previous studies by Burk *et al.* (*25*)] the average $Q_{CO_2}^{N_2}$ of pooled slices from 3 actively growing H-P melanomas (51 days after transplantation) weighing about 1.4 grams each, from "unstressed" C mice, was 1.16 (no effect of added insulin) at 37° C. In KRBC plus 0.6 percent glucose the average $Q_{CO_2}^{N_2}$ of the same tumors was 1.73. The difference between the values obtained in the present investigation and those in the report of 7 years ago ($Q_{CO_2}^{N_2}=11.0$) cannot be explained with certainty. If they are due to changes in the H-P melanoma, they are not associated with grossly recognizable alterations in the morphology or growth characteristics of the tumor. However, the H-P tumors previously studied were grown mainly in C3H mice (*25*), while those used here were grown in either C or DBA mice. It should also be noted that $Q_{CO_2}^{N_2}$ values for the S91 melanoma reported in the present paper are higher, on the average, than those (*25*) previously reported (20 *versus* 11.8), whereas the present values for the S91A tumor are lower (11.5 *versus* 14.8). Nevertheless, it is only in the H-P melanoma that the order of magnitude has been appreciably different.

Effect of Stress, Insulin, and Testosterone

As in the case of S91 melanoma, H-P slices from temperature-stressed mice showed a depressed glycolysis relative to slices of tumors from mice maintained at 25° C. Thus in four experiments with tumors from stressed (35° C. for 46 to 116 hours) and "unstressed" (25° C.) mice, the average $Q_{CO_2}^{N_2}$, at 34 to 40° C., in the absence of insulin, at approximate glycolytic equilibrium, was 0.94 (0.57 to 1.49) for the 35° tumors, and 1.90 (0.88 to 2.66) for the 25° tumors. In the presence of insulin the respective values were 0.78 (0.41 to 1.23) and 2.04 (1.19 to 2.64). Thus, insulin inhibited glycolysis in the 35° tumors and caused a small average stimulation in the 25° tumors. At first glance, these results might suggest that the glucose metabolism of this tumor is not significantly sensitive to insulin. There are several lines of evidence, however, that suggest that these tumors are sensitive to the type of insulin: anti-insulin mechanism which operates in the S91 tumors. These can be briefly summarized as follows: (*a*) The shapes of the $Q_{CO_2}^{N_2}$ time curves in the H-P melanoma, at various temperatures of *in vitro* incubation, resemble those of the insulin-sensitive S91 melanoma rather than those of the hormone-insensitive Krebs-2 tumors. (*b*) Exposure of the H-P melanoma-bearing animals to 35° C. results in a marked decline in the subsequently measured $Q_{CO_2}^{N_2}$, as in the case of S91 melanomas, and in contrast to the Krebs-2 tumors. (*c*) Stimulation of *in vitro* glycolysis by exogenous insulin has been obtained repeatedly in H-P melanoma, but unlike the S91 tumor this has occurred chiefly in H-P tumors from "unstressed" mice, and the effect is generally less pronounced than in the S91 melanoma.

In the S91 melanoma, severe temperature stress resulted in a marked lowering of tumor slice glycolysis which, in such extreme cases, could not

always be reversed by insulin (*1*). The inability of exogenous insulin to reverse the temperature-stress-induced inhibition of glycolysis in S91 melanoma was further accentuated by lowering the *in vitro* incubation temperature to 20° C. and by addition of exogenous testosterone. In many respects, the "unstressed" H-P melanomas (25° tumor, table 9) behaved like "heavily stressed" S91 tumors. In addition to the very low $Q_{CO_2}^{N_2}$ in the H-P melanoma, the average percent inhibition by testosterone was much lower in general in H-P than in the S91 melanomas (only 1 to 23% at 20° C. and 5 to 29% at 34 to 40° C.). In this respect, H-P tumors resembled S91 melanomas following extreme exposure to temperature stress (see text-fig. 13). While there may be another explanation, such behavior suggests that in H-P tumors there is already a relative saturation with endogenous steroid so that further *in vitro* addition of such compounds has little effect. Against this argument stands the fact that in heavily stressed H-P melanomas complete inhibition of glycolysis at 20° C. did not occur, even in the presence of exogenous testosterone. Under such conditions the $Q_{CO_2}^{N_2}$ of S91 tumors was sometimes reduced to zero. It should be noted, however, that all available evidence from studies in nonmalignant tissues (see Discussion) points to the anti-insulin mechanism being a multifactor one in which a pituitary factor, or factors, may also play a limiting role. Certain other anomalies have been repeatedly observed in the H-P melanoma. These are illustrated in table 9, which compares the $Q_{CO_2}^{N_2}$'s of a 25° H-P melanoma with those of a 35° tumor (68 hours' exposure) of the same transplant generation. The average $Q_{CO_2}^{N_2}$ values for the 25° tumor were higher (*ca.* 100%) than the corresponding 35° tumor values in all cases, except during the first 68 minutes of incubation at 20° C. in the absence of insulin (insulin columns) or in the presence of testosterone (either plus or minus insulin). For the first 68 minutes at 20° C. there was a marked response to insulin

TABLE 9.—$Q_{CO_2}^{N_2}$ *values of Harding-Passey melanomas from temperature-stressed and "unstressed" strain C mice*

Temperature (*time in minutes*)	$Q_{CO_2}^{N_2}$ 25° tumor				$Q_{CO_2}^{N_2}$ 35° tumor (*68 hours' exposure*)			
	−Testosterone		+Testosterone		−Testosterone		+Testosterone	
	−Insulin	+Insulin	−Insulin	+Insulin	−Insulin	+Insulin	−Insulin	+Insulin
20° C.								
28–68	0. 42	0. 90	0. 29	0. 82	0. 46	0. 32	0. 93	0. 97
73–218	0. 31	0. 42	0. 30	0. 24	0. 17	0. 21	0. 21	0. 19
34° C.								
3–18	1. 95	2. 33	1. 62	0. 67	1. 15	0. 43	0. 38	0. 57
18–148	0. 76	1. 06	0. 78	0. 66	0. 50	0. 41	0. 38	0. 33
148–1023	0. 53	0. 72	0. 52	0. 45	0. 33	0. 26	0. 20	0. 22
1023–1113	0. 29	0. 44	0. 38	0. 30	0. 21	0. 15	0. 07	0. 19
Final *p*H	7. 38	7. 30	7. 37	7. 40	7. 43	7. 47	7. 52	7. 51

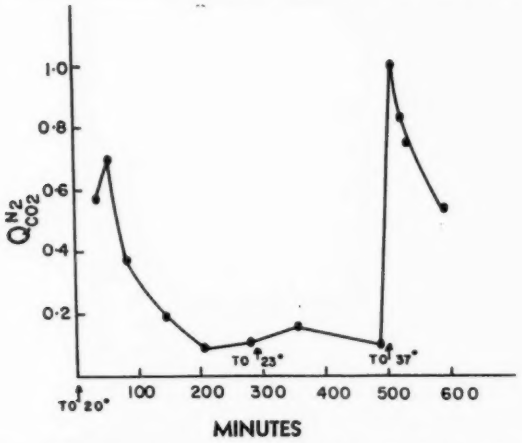
(114%) in the 25° tumor, although by 218 minutes the percent stimulation had diminished to only 35. In the presence of testosterone, insulin inhibited glycolysis 20 percent. In marked contrast, the effect of insulin in the 35° tumor was predominantly inhibitory, and testosterone produced a transitory stimulation in acid production at 20° C. Similar, though less marked, transitory stimulations by testosterone have been observed in other H-P tumors from heat-stressed animals. Such effects have occasionally been observed in S91 melanomas (see text-fig. 10). While transitory critical concentrations of testosterone might be stimulatory rather than inhibitory, this explanation is hard to reconcile with the fact that such effects occur in tumors from stressed animals (table 9) where presumably the endogenous concentration of anti-insulin steroid is high. However, again it should be recalled that available evidence points to a multifactor system in which both relative, as well as absolute, concentrations of the component factors may be of importance. In this connection, it is of interest that the stimulation of protein anabolism by testosterone seems to depend on the presence of insulin (*26*). The maintenance of a critical intracellular concentration of the steroid *in vitro* presents a special problem since testosterone can be metabolized (*27*) by certain tissues, although this has not been established for the malignant tissues concerned in the present investigation. No explanation for the inhibitory effects of insulin, which occur under certain conditions, can be advanced at the present time. The phenomenon has been repeatedly observed in this tumor by us and by Wolff (unpublished data) working in this laboratory.

Human Melanoma

Our studies of human melanoma reported here were restricted to several tumors removed from one patient during the course of surgical excisions of metastatic foci. The data, though necessarily limited, indicate similarities to the mouse melanomas in that there was evidence of a pronounced anti-insulin inhibitory mechanism. These human tumors were unusual for melanoma in that they were relatively slow-growing and heavily infiltrated with fibrocytes. Text-figure 21 shows the $Q^{N_2}_{CO_2}$ time curves (at 20 and 37° C. with 0.6% glucose) obtained from a 200 mg. sample from a metastatic nodule on the patient's back, close to the site of the original tumor. This tumor was very heterogeneous with respect to distribution of pigmented tumor cells and infiltrating connective tissue. The steep decline in rate (text-fig. 21) to equilibrium at a low level ($Q^{N_2}_{CO_2}=0.1$), together with the large temperature coefficient, suggested the presence of a pronounced glycolytic inhibitor mechanism of the type observed in mouse melanoma. In other ways the behavior of this tumor was similar to that of H-P melanoma from heavily stressed mice where exogenous zinc insulin also depressed glycolysis.

A second series of operations was performed about 10 days later to remove the only known remaining metastatic foci. Table 10 summarizes metabolic determinations in some of the tumor tissue removed. Sixty to 130 mg. samples (wet weight) of tissue were incubated in KRBC with

0.6 percent glucose (2.15 ml. total volume) and 27 *gamma* (0.7 unit) of zinc insulin per ml. The nodule removed from the patient's shoulder was histologically similar to the older metastatic nodule from the back with the exception that slightly more melanized tissue was present. The chest nodule was the farthest removed from the original site of the mela-



TEXT-FIGURE 21.—$Q^{N_2}_{CO_2}$ time curves of human melanoma slices (200 mg. wet weight per vessel) in KRBC with 0.58 percent glucose (3.2 ml. total volume) at three successive temperatures of incubation.

TABLE 10.—*Glycolytic activities in human melanoma*

Temperature (*time in minutes*)	Shoulder tumor			Chest tumor		
	$Q^{N_2}_{CO_2}$		Percent insulin effect	$Q^{N_2}_{CO_2}$		Percent insulin effect
	−Insulin	+Insulin		−Insulin	+Insulin	
20° C.						
15–20	0. 88	0. 45	*−49*	1. 67	3. 56	*+113*
80	0. 20	0. 22	*+10*	0. 20	0. 59	*+195*
245	0. 11	0. 12	*+9*	0. 17	0. 16	*−6*
	To 37°	*at 248*				
37° C.						
250–253	19. 39	8. 87	*−54*	25. 92	28. 14	*+9*
255	10. 84	8. 97	*−17*	18. 33	20. 00	*+9*
260	4. 09	2. 72	*−34*	4. 88	4. 44	*−9*
295	0. 89	0. 74	*−17*	0. 59	1. 14	*+93*
320	0. 51	0. 58	*+13*	0. 33	0. 53	*+61*
Tip Neotetrazolium						
335	0. 80	0. 85	*+6*	0. 82	1. 48	*+80*
355	0. 81	0. 81	*0*	0. 61	0. 89	*+46*
500	0. 50	0. 58	*+16*	0. 37	0. 46	*+24*
530	0. 62	0. 68	*+9*	0. 61	0. 89	*+46*
Final *p*H	7. 49	7. 53		7. 62	7. 58	

noma. Again the temperature coefficients were large, and, because of the rapid decline in rate, the precise maximum $Q_{CO_2}^{N_2}$ values are uncertain. It seems clear, however, that the shoulder tumor (table 10) was more under the influence of the glycolytic inhibitor mechanism than the chest tumor. This was evidenced in the shoulder tumor by (*a*) the lower initial $Q_{CO_2}^{N_2}$ at 20° C., (*b*) the lower equilibrium values (see table 10, 245 minutes), (*c*) the *inhibiting* effect of insulin, and (*d*) the lower observed maximum $Q_{CO_2}^{N_2}$ values immediately after transfer to 37° C. In contrast to the back and shoulder tumors, the highest rates in the *chest* tumor occurred in the presence of exogenous insulin—in this respect paralleling the situation in H-P mouse melanoma (see table 10, 25° tumor). These data, although limited, nevertheless indicate that insulin:anti-insulin regulation of glucose metabolism may play an important role in human melanoma growth as it appears to do in mouse S91 melanoma. Further observations of human tumors will be made as opportunities are presented.

Discussion

Role of Insulin in Tumor Metabolism

In spite of the important role of insulin in regulating glucose metabolism there has been relatively little of a definitive character published concerning the importance of this hormone in tumor metabolism *per se*. Ryer (*28*) found that when rabbits bearing the Brown-Pearce epithelioma were given repeated injections of insulin, both liver and tumor (as measured subsequently *in vitro*) showed increased rates of oxygen consumption. Administration of thyroxine and insulin together also resulted in hypernormal Q_{O_2} values in the tumor, whereas insulin (given *in vivo*) markedly reduced aerobic glycolysis in the tumor and thyroxine, as it became increasingly effective, tended to counteract this action of insulin. Ryer concluded that, while both hormones tend to increase the oxidative capacity of the tumor, each is functioning at a different level in the oxidative pathways. He obtained 50 to 80 percent repression of metastases in animals treated with these hormones. These results contrast with the present findings in melanoma where an increase in endogenous *anti*-insulin factors (as in temperature stress) is associated with a marked suppression of tumor glycolysis and growth. However, administration of insulin *in vivo* might lead to a temporary retardation in growth, as a consequence of a lowered glucose supply to the tumor resulting from induced hypoglycemia. Carrie and Ham (*29*) found that a transplantable sarcoma grew as well in alloxan-diabetic rats as in normal rats and that the tumor had no effect on the glycemia. However, no information was presented as to the sensitivity, or lack of sensitivity, of this tumor to anti-insulin hormones. If the tumor was insensitive to anti-insulin hormones, the tumor presumably would not be dependent on insulin for growth and might, in fact, even be benefited by the hyperglycemia of the diabetic animals.

It was previously suggested (*1*) that insulin might play a role in tumor growth. This seemed likely because of the marked parallelism between

insulin-reversible lowering of glycolysis and the suppression of tumor growth which occurred following exposure of the host-tumor system to 35° C. That insulin acts in a special way to stimulate growth of nonmalignant tissue seems well established from the work of Salter and Best (*30*) and Lawrence, Salter, and Best (*31*). As Young [(*32*) p. 21] has pointed out " . . . the balance of evidence suggests . . . that insulin may play a much more important role in protein metabolism than has hitherto been suspected . . ." and he suggests further (p. 22) that " . . . it may well be that protein anabolism is particularly under control of insulin secreted by the pancreas, and that any direct influence of growth hormone on protein synthesis is of minor significance only." In this connection it is of interest that insulin has been reported to stimulate cell growth in tissue culture (*33*, *34*). Of particular interest are the findings of Bullough (*35*, *36*) that pituitary growth hormone inhibits mouse-ear epidermal mitosis *in vitro*, and that insulin can partially reverse this effect (85% inhibition without insulin, 70% with 50 *gamma* insulin added). Recently the protein anabolic effect of testosterone has been related to insulin (*26*) although the precise nature of the relationship has not been defined.

The question arises as to whether the insulin:anti-insulin regulation of cell metabolism and growth, which seems to operate in the melanomas that we have studied, is unique for this particular group of neoplasms and therefore of somewhat restricted interest. The lack of hormone sensitivity of the Krebs-2 carcinoma (either as a solid or ascitic tumor) might seem to favor the more restrictive view. However, there are several lines of evidence which suggest that this should not be the case. These are: (*a*) the fact that diverse types of nonmalignant tissue appear to have an insulin-sensitive glucose metabolism; (*b*) the evidence that progressive anaplasia (as in conversion of S91 to S91A types) is associated with loss of sensitivity to anti-insulin hormones; and (*c*) the mounting evidence that insulin plays a key role in growth regulation.

Insulin Action in Nonmalignant Tissues

Insulin apparently plays a positive role in the regulation of carbohydrate metabolism in a number of different cell types, although the literature is conflicting at a number of points. There seems to be rather general agreement that the metabolic defects of depancreatization lie (at least partly) in the rate of glucose uptake by skeletal muscle, cardiac muscle, liver, and adipose tissue [*cf.* Levine and Goldstein (*37*) for review]. Insulin has also been shown *in vitro* to exert positive effects in lactating mammary gland tissue (*38*), epithelial tissue (*35*, *36*), and brain (*14*, *39*). The high affinity of kidney for insulin (*40*), together with data of Wolff (unpublished) make it likely that this tissue is also insulin-sensitive. Studies of $Q_{CO_2}^{N_2}$ time curves in brain, liver, and kidney (unpublished data) suggest that mechanisms regulating utilization of glucose similar to those occurring in the S91 melanoma occur in these tissues. On the other hand, in the Krebs-2 tumor they seem to be nonoperative.

If it is true that insulin:anti-insulin mechanisms regulate glucose utilization in diverse types of normal tissues, it seems most unlikely that such systems would not operate in tumors of various types—at least in the earlier phases of tumor history. This is made the more likely by the universal occurrence of an active glucose metabolism in tumors. In this connection it should be noted that the reported lack of insulin requirement, or binding, by brain or other tissues (*41*, *42*) may merely be the result of a relatively high saturation with, and retention of, insulin by these tissues so that experimental changes in the amount of the circulating hormone produce little apparent effect.

Insulin : Anti-Insulin Hormones and Tumor Autonomy

Conversion to the malignant state involves the development of varying degrees of autonomy (*43*, *44*); however, it has been abundantly proved that varying degrees of specific dependence on hormones elaborated by the host exist throughout the spectrum of mammalian tumors. The present data, obtained with the group of neoplasms that were derived by transplantation from the original S91 DBA mouse melanoma, show that cellular variation, which is partly expressed as a loss in tyrosinase activity (*15*, *19*) and as an enhanced capacity to grow in a foreign host strain (*16*, *21*) is also associated with a greatly lowered sensitivity to the hormones of the anti-insulin inhibitor complex. In this case, developing autonomy is associated with a loss of sensitivity to a basic hormonal mechanism which probably operates in the regulation of glucose metabolism in many normal cell types. Thus the development of malignancy in the first place may depend in part upon a sufficient loss in sensitivity to this hormonal mechanism (insulin:anti-insulin) which regulates the first stages of glucose utilization. Further cellular modification results in still further loss in sensitivity to these particular hormones, to the extent that the tumor can continue to grow even in the presence of unusually high levels of the anti-insulin factors (as, *e.g.*, in temperature-stressed mice). In confirmation of this interpretation of the S91–S91A tumor interrelationship, Hunter (*17*) has recently obtained strongly amelanotic derivatives of S91 tumors maintained at host-stressing temperature. It seems likely that in this case a change in host endocrine balance resulting from temperature-induced stress has acted as a "natural selector" of hormone-resistant cell types. It is noteworthy that these derivatives have metabolic patterns strongly resembling the S91A amelanotic derivative of the S91 melanoma. The Krebs-2 carcinoma, either as an ascites or solid tumor, shows little, if any, growth sensitivity to temperature stressing of the host-tumor system, and likewise presents a metabolic pattern that is very insensitive to insulin or anti-insulin factors. The lack of host specificity and the morphologic characteristics of this tumor, together with its long history of transplantation (*6*), are in line with the concept that the Krebs-2 tumors are highly anaplastic, although this has not been definitely established. The present data raise the question as to the

possible interrelationships which may exist between hormonal *dependence* of growth, hormonal *suppression* of growth, and hormonal *independence* of growth. Through variations in balance between different hormones and/or their absolute concentrations, metabolism and growth might be either stimulated or suppressed. Thus, hereditary (in a cellular sense) sensitivity of a particular tumor to a particular hormonal complex (*e.g.*, insulin : anti-insulin system) might be expressed as either hormone dependence or, conversely, as susceptibility to inhibition by the same hormones. In this connection it would be of interest to know to what extent the observed insensitivity *in vitro* of the hexokinase reaction of certain tumors (Ehrlich ascites and Gardner's lymphosarcoma) to adrenal steroids (*45*) could be explained on the basis of cellular modification of the type observed in the S91–S91A series of tumors. The evidence that relevant enzymic-hormonal systems are mitochondrially localized (*13*, *20*, *46*) should be considered in relation to the evidence that mitochondrial systems have the property of intrinsic heredity and are subject to both intracellular selection and intercellular segregation (*47*).

Stress and Insulin : Anti-Insulin Systems

The present data show that exposure of the host-tumor system to stressful conditions greatly accentuates the activity of the anti-insulin glycolytic inhibitor mechanism, which, however, appears to operate also in "unstressed" mice, though less effectively. The demonstrated partial similarities and additivities between the effects of *in vivo* temperature stress and *in vitro* addition of testosterone on tumor slice glycolysis offer definite clues as to the nature of the mechanism involved, especially when viewed in the light of insulin reversibility. An extensive literature relating conditions of stress to carbohydrate metabolism has developed in recent years. While an extensive review cannot be attempted here, certain aspects are pertinent to the present investigation.

It has been well established that the pituitary-adrenal axis is especially involved in the stress syndrome, and the secretions of such organs are involved in the regulation of glucose metabolism [*cf.* (*10*, *48*) for reviews]. It seems clear that activation of the adrenal cortex is intimately involved (*48–51*) in this process, although the role of cortisone, or other corticoids, may be only an indirect one (*48*). Of particular interest are the observations of Ingle (*52*) that estrogen (diethylstilbestrol) has a diabetogenic effect which is not mediated by a change in the secretory activity of the adrenal cortex, but apparently depends on the presence of cortical hormones for its expression. Drucker *et al.* (*53*) have observed in man that stresses of acute febrile illness and surgery diminish tolerance for glucose but not fructose. Hinkle and Wolf (*54*), as the result of a survey of life histories of persons with diabetes mellitus, concluded that life experiences (stress) are of great importance in the onset and course of the disease. Vaccari and Rossanda (*55*) studied blood glycolysis during the alarm reaction and after treatment with adrenal hormones and ACTH. They

observed, during the alarm phase of stress, an initial accentuation of glycolysis followed by a depression and a final reversion to normal during the "countershock" phase. In very strong stresses glycolytic depression prevailed. ACTH in a large dose induced a picture similar to heavy stress. Bullough (*36*) has implicated stress and associated hormonal changes involving insulin in the suppression of mitosis in mouse ear epithelium.

It has been well established that stress, such as exposure to high or low temperatures, can result in marked changes in adrenal and pituitary activities, and as a direct or indirect result of this, to changes in the insulin-sensitive regulation of glucose metabolism by normal tissues. So far as we are aware, the present paper and the ones preceding (*1*, *3*) are the first reports in mammalian cancer literature of the effects of stressful conditions on the insulin-sensitive mechanism which regulates glucose metabolism and, presumably, growth. While the existence of a state of stress may be assumed in mice exposed 12 hours or more to 35° C., or in mice bearing large tumors which are unable to maintain homeostasis during exposure to 20° C., we have not attempted to use such criteria of stress as changes in quantity of circulating eosinophils, changes in adrenal weight, depletion in adrenal cholesterol and ascorbic acid, excretion of steroids, etc. Rather we have considered the degree of insulin-reversible lowering of glycolysis in the tumor as an indicator of changes in host endocrine balance which result from exposure of the host-tumor system to the noxious influence. In this connection it should be noted that a tumor-bearing animal is already in a state of stress or incipient stress as a result of malignant disease. However, in our experiments, it is significant that exposure of mice bearing very small S91 melanomas (less than 0.5 gm.) to 35° C. nevertheless resulted in inhibitions of tumor growth and metabolism that were just as pronounced as those occurring in similarly treated mice bearing large (10 gm. or more) melanomas (*1*). As previously pointed out (*1*), exposure of mice with small to moderate-sized melanomas to 20° C. normally results in a tumor response which is just the opposite of that produced by exposure to 35° C. Accentuation of insulin-reversible lowering of tumor glycolysis in 20° mice occurs only when the tumors are very large. In this case, it appears that a temperature which is not noxious to mice with small to moderate-sized tumors becomes highly stressful when the mice bear very large tumors. The latter type of animal might be regarded as "doubly stressed." Additional studies (unpublished) have indicated that insulin sensitivity can be accentuated by cold even in small melanomas (1 gm.), but only following extreme exposure (alternate wetting and exposure to subzero temperatures). These results are interpreted as supporting the concept that the observed modifications in tumor metabolism and growth which are associated with the exposure of mice to a temperature of 35° C. are reflections of changes in host endocrine balance involved in a general adaptation syndrome (*48*, *56*). As such, certain types of tumors become sensitive bioindicators of alterations in host endocrine balance. Conversely, the effects of host stress on tumor metabolism and growth permit a considerable evaluation of the sensitivity of the malignant cells to certain types of hormonal influence.

Mechanism of Insulin Action

In general, our data, which report rates of anaerobic acid production from glucose, are most consonant with the views of the Cori school (*2, 10, 11, 56*), which propounds that insulin acts to relieve an inhibition of the hexokinase reaction resulting from the mutual action of protein hormones from the anterior pituitary and steroids of the adrenal cortex, although other steroids may be involved. However, since Krahl [(*10*) p. 27] has pointed out that ". . . the glucose uptake of muscle is in part under an insulin-reversible inhibitory influence from the pituitary and adrenals and in part under an inhibition which persists even when both of these glands are removed . . ." caution should be exercised in attributing a direct or exclusive action to the adrenal steroids in inhibition of glycolysis. This is particularly true when the evidence for the indirect role of the adrenal cortex in producing stress effects on nitrogen and carbohydrate metabolism is considered (*48, 49*).

The recent findings of Bornstein and Park (*11*) are particularly suggestive in relation to the present findings in melanoma. These authors have shown that the serum of alloxan-diabetic rats inhibits *in vitro* glucose uptake by the diaphragm of normal fasted rats. The inhibition is reversed *in vitro* by insulin. On the other hand, serum from adrenalectomized or hypophysectomized rats does not inhibit glucose uptake. The lost inhibitory property of the serum of hypophysectomized rats can be restored by injection of growth hormone plus cortisone but not by either substance alone. Growth hormone and cortisone are ineffective *in vitro*. Further work (*12*) showed that the inhibitor was associated with a lipoprotein fraction. We have obtained a small insulin-reversible lowering of glycolysis in S91 melanoma with cortisone, but growth hormone had no effect either alone or in combination with steriod (unpublished data). We have not yet tested these substances *in vivo*.

While there is considerable literature supporting the view that insulin acts by relieving inhibition of intracellular enzyme [*cf.* Krahl (*10*)], this has not been accepted in all quarters. Levine and Goldstein (*37*) have suggested that insulin causes changes in the functional structure of the cell membrane which permit more rapid entrance of glucose into the cell. More recently, Park (*58*) has presented further evidence that insulin (*in vivo*) increases by 2 to 5 times the intracellular glucose content of muscle from eviscerated rats. These results, which show that insulin speeds up transfer of glucose into the cell at rates which exceed phosphorylation, might suggest that the real site of action is the cell membrane rather than internal enzymic loci. However, in addition to the demonstrated effects of insulin on cell-free hexokinase preparations (*2, 57*), it has also been shown by Elgee *et al.* [(*40*) p.370] that 15 minutes after intravenous injection of iodine-labled insulin into rats it becomes *intracellularly* localized in liver, kidney, and muscle. Thus insulin became "distributed in the cytostructural elements of the liver in a characteristic manner and fixation on the subcellular particles was shown to depend on cellular integrity." These results are of special interest in relation

to the findings of duBuy *et al.* (*13*) and Hesselbach and duBuy *(46)* that, contrary to the often expressed opinion that cellular glycolysis is localized in the interparticulate ("supernatant" of subcellular fractionations) cytoplasm, these activities are in reality concentrated (although not exclusively) in the cytoplasmic particulates (mitochondria and subvisible particles).

Chemotherapeutic Implications

Possible Increase in Sensitivity to Oxygen Deprivation

At this point we wish to make some comments on the significance of the present findings with respect to possible applications in the field of chemotherapy. As Warburg *et al.* have pointed out [(*59*) p. 255 *et seq.*], tumor cells can withstand oxygen deprivation if they receive an adequate supply of glucose; conversely, they can withstand a lack of glucose if adequate oxygen is present. The ability of glucose to protect *in vivo* against the lethal effects of anoxia has been well established (*60*). The present results with melanoma have made it clear that the tumor cells can retain their glycolytic capacity *in vitro* in the absence of oxygen at an almost undiminished rate for 24 to 48 hours provided sufficient glucose and buffering capacity are present. The data also show that the action of the anti-insulin inhibitor mechanism is relatively much more pronounced at low glucose concentrations (0.05 to 0.10%) than at a high level of glucose (0.60%). Since normal tissues (*e.g.*, brain and kidney) are much less affected by the stress-accentuated inhibitor mechanism than the sensitive tumors (possibly because normal tissues are relatively saturated with both insulin and anti-insulin hormones under normal conditions), it would appear that a means exists here for *selectively* lowering the glucose-utilizing capacity of the cancer cells. It is of particular interest in this connection to learn to what extent this might modify the vulnerability of the malignant cells to oxygen deprivation *in vivo*. The latter condition may occur naturally in certain areas of the tumor due to inadequacies in vascularization or might be brought about experimentally by relatively specific chemical blocking at some point in the aerobic metabolism of the tumor cell.

Interaction with Acetylpodophyllotoxin-ω-Pyridinium Chloride

The results of Waravdekar *et al.* (*61*) and Algire (*62*) are of interest in the foregoing connection, since they suggest the possibility of providing, directly or indirectly, a considerable degree of selective oxygen deprivation in tumors. It should be noted that we have found (*63*) that a podophyllotoxin derivative (NCI-3022 acetylpodophyllotoxin-ω-pyridinium chloride obtained through the courtesy of Dr. J. Leiter) also inhibits the *in vitro* anaerobic glycolysis of S91 melanoma 50 to 70 percent at 20° C. This inhibition, like that caused by testosterone, is markedly reversed by elevating the incubation temperature to 37° C. and by exogenous insulin. Presumably, NCI-3022, like the steroid, *acts by potentiating the endogenous anti-insulin mechanism* in the melanoma, since anaerobic glycolysis of the

hormone-insensitive Krebs-2 ascites tumor is not affected by this podophyllotoxin derivative at either 20 or 37° C.

Summary

Employing melanoma S91 as bioindicator, and using newly developed *in vitro* techniques of metabolic analysis involving determinations of glycolytic rate ($Q_{CO_2}^{N_2}$) time curves at successive and widely different temperatures of incubation, it is shown that glycolysis in this tumor is under the control of a stress-modifiable insulin : anti-insulin mechanism that regulates glucose utilization. The new methodology includes determination of changes in the rate-time curves at constant subnormal temperatures (*e.g.*, 20° C.) or following a shift in temperature (*e.g.*, from 20 to 40° C. or *vice versa*). The effects of exogenous insulin, testosterone, and glucose concentration on the $Q_{CO_2}^{N_2}$ time curve are especially considered. When the temperature of *in vitro* incubation is suddenly changed (*e.g.*, from 40 to 20° C., or *vice versa*) the new $Q_{CO_2}^{N_2}$ time curve indicates the occurrence of a gradual and reversible readjustment of balance within the insulin: anti-insulin system.

In vitro addition of insulin or raising the glucose concentration tends to counteract glycolytic inhibitor action and therefore raises the level of final glycolytic equilibrium. Conversely, prior *in vivo* exposure of the host-tumor system to high temperature stress (35° C.), lowering the glucose concentration, or addition of certain steroids (*e.g.*, testosterone, but not cholesterol), favors inhibitor action, and therefore lowers the level of final glycolytic equilibrium. Development of severe alloxan diabetes (blood sugars 400 to 600 mg. %), while associated with some increase in the *in vitro* sensitivity of the tumor to insulin (average insulin effect 23% compared to 10% for untreated tumors), has much less effect than *in vivo* temperature stress (average insulin effect 50%).

The glycolysis-stimulating (insulin) and the inhibiting (anti-insulin) components of the insulin:anti-insulin system respond differently to changes in the temperature of incubation. Increasing the temperature shifts the balance toward insulin action—lowering the temperature shifts the balance toward anti-insulin action.

These techniques of analysis constitute a powerful tool for *in vitro* determination of metabolic sensitivities of tumors and certain normal tissues to glycolysis-regulating hormones. By application of these techniques it can be shown that cellular modification (as represented by the S91A and partially amelanotic derivatives of the S91 melanoma) can be associated with pronounced changes in the form of the $Q_{CO_2}^{N_2}$ time curves, indicating decreased sensitivity of glycolysis to the stress-modifiable insulin:anti-insulin hormonal system. In the same manner the Krebs-2 ascites tumor was found to be virtually insensitive to this system. Growth capabilities in mice under stress of the S91, S91A, and Krebs-2 (solid form) tumors (elsewhere reported) are strongly correlated with the metabolically indicated anti-insulin sensitivities as determined by the described methods.

Metabolic determinations in Harding-Passey mouse melanoma and human melanoma indicate the presence of a pronounced insulin:anti-insulin regulation of glucose metabolism.

The implications of these findings and newly developed methods of biological resolution with respect to problems of chemotherapy are discussed.

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Differential Effects of Temperature on the Growth of Certain Transplanted Tumors in Strain DBA Mice[1]

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Experimental data reported from this laboratory in 1953 (*1*) indicated that a relationship exists between temperature-induced, insulin-reversible lowering of anaerobic acid production *in vitro* and tumor growth. Further work (*2*) has demonstrated that the effects of environmental temperature on tumor metabolism are definitely due, in part, to accentuation of an anti-insulin inhibitor mechanism. Since the insulin-reversible lowering of glycolysis was found to result, under certain conditions, from exposure of tumor-bearing animals to low temperatures (20° C.) as well as to high temperatures (35 to 40° C.), the role of a stress mechanism is indicated (*2*). This view finds additional support in the fact that during stress, endocrine balances are disturbed, particularly those balances concerned with the interaction of adrenocortical and pituitary secretions (*3–6*), and these factors are also known to be involved in insulin regulation of glucose metabolism (*7–9*). Since metabolic studies have shown (*2*) that the Krebs-2 carcinoma (*10*), S91A amelanotic melanoma (*11, 12*), and S91 melanoma (*13*) differ greatly with respect to their sensitivity to temperature-induced, insulin-reversible lowering of glycolysis over host mechanisms (Krebs 2 being least sensitive, S91 most sensitive, and S91A intermediate between the two), it seemed desirable to study the comparative growth rate of these tumors at controlled temperatures. This paper reports effects of environmental temperature on the growth of these three tumors, as well as a manometric study of the effect of exogenous hormones on the anaerobic acid production of tissue from a derived S91 melanoma that is not sensitive to high environmental temperature.

Materials and Methods

Young adult male DBA mice of unknown subline (Poiley, NIH)[4] from 2 to 5 months old, and weighing 20 to 25 grams per mouse, were given

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[4] The S91 melanoma has been maintained in both DBA/2 JN and the Poiley strain of DBA with equal results as displayed in rate of growth, hormone sensitivity, and response to high temperature. The Poiley mice were used in this experiment because they are more plentiful and seemingly more hardy than the DBA/2 JN strain.

intramuscular inoculations of small, uniform pieces (*ca.* 1 mm.^{3}) of the S91 melanoma, S91A amelanotic melanoma, and all derivatives of the S91 used for this study, using 14-gauge trocars. The Krebs-2 carcinoma in ascites form was inoculated, in amounts of 0.3 ml. of freshly drawn ascitic fluid per mouse, intramuscularly by hypodermic syringe with a 20-gauge needle. Inoculations were made into the right thigh muscle in all cases. A maximum of 6 animals was placed in a cage to avoid the stressing effects of crowding (*14*), especially upon those animals held at the higher temperature. In this connection, animals were kept in plastic cages measuring 11 × 6⅞ × 4¾ inches with perforated metal tops. During the course of an experiment, half of the inoculated mice were placed in a controlled temperature box at 35° C. ± 1° while the other half were kept in a similar box at 25° C. ± 1°. Both control and experimental animals were kept in the dark and received Purina chow and water *ad libitum*. Mice in each cage were numbered by ear punch.

For the manometric experiment, anaerobic conditions were provided by gassing with a mixture of 95 percent N_2 and 5 percent CO_2 which had been passed along with a small amount of H_2(*ca.* 1%), over hot copper to remove traces of O_2. The medium was Krebs-Ringer's, 0.04 *M* $KHCO_3$. The concentration of dextrose used was 0.625 percent, a concentration which gives maximal rates of anaerobic acid production in melanoma slices without significantly inhibiting respiration (*1*). Acid production was measured in terms of CO_2 released from the bicarbonate-buffered medium.

The insulin preparation used was zinc insulin crystals (Lilly, Lot No. 535664). The crystalline zinc insulin was dissolved in 0.025 *N* NaOH and the *p*H immediately adjusted to about 7.0. This method of preparation gave a clear solution (*1*). A Schering preparation of testosterone was used. The androgen was dissolved in 2 ml. of absolute alcohol and the volume was made up to 12 ml. with distilled water. All vessels in the manometric experiment received the same percentage of alcohol as a control.

The symbol $Q_{CO_2}^{N_2}$ has been used to designate microliters of acid in terms of CO_2 produced per mg. initial dry weight per hour in the absence of oxygen.

Since the tumors were transplanted intramuscularly, it was impossible to measure the entire tumor, so rate of growth was determined by measuring the accessible portion of the tumors in centimeters in three dimensions. It was recognized that, since a tumor is ellipsoidal in shape, there would be some error in this method, but it was found that the error involved does not preclude determining accurate size differences on a relative basis.[5] The product of these dimensions was determined and used as the tumor volume in cubic centimeters.

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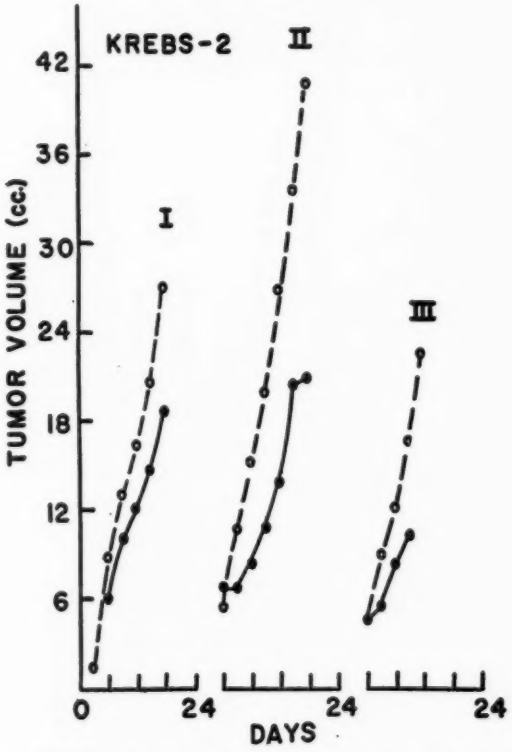
[5] The measured volumes, in cubic centimeters, of several S91 tumors of different sizes were obtained using caliper measurements of these tumors in three dimensions. These volumes were multiplied by the density for the S91, which had been previously determined by water displacement. The theoretical mass thus obtained was compared to the actual mass of the tumors. The range of the standard error was from 0.4 gm. for tumors with an average measured volume of 0.4 cc. to 2.5 gm. for tumors with an average measured volume of 13 cc., which was found to be adequate for this investigation.

Experimentation and Results

Krebs-2 Carcinoma

The first tumor studied was the Krebs-2 carcinoma in solid form. Ten ml. of Krebs-2 ascitic fluid was withdrawn from a BALB/cAnN mouse and 0.3 ml. was inoculated intramuscularly into each of 12 adult male DBA mice. Six mice were placed in each of two cages, weighed and placed at the experimental temperatures 1 hour after inoculation. The growth curves for the tumors at the different temperatures are presented as I in text-figure 1, which shows that high temperature had very little effect on the growth rate of the Krebs 2. By the 15th day after transfer and exposure to controlled temperatures, 3 of the animals held at 35° C. were dead while there were no deaths at 25° C. On the 18th day of the



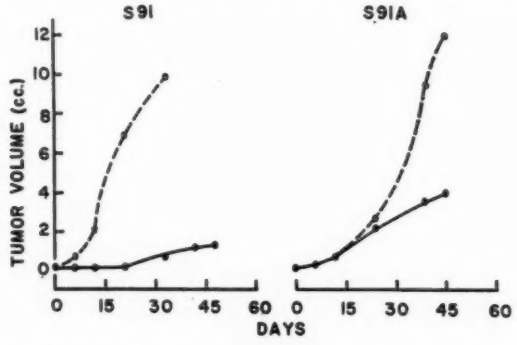
TEXT-FIGURE 1.—Growth rate of the Krebs-2 carcinoma (solid form) in strain DBA male mice at 35° C. (*solid line*) and 25° C. (*dash line*), in three experiments, expressed as average tumor volume plotted against time. I—Half of the mice placed at 35° C., 1 hour after inoculation; II—half of the mice placed at 35° C., 3 days after inoculation; III—half of the mice placed at 35° C., 6 days after inoculation. All animals placed at 25° C. immediately following inoculation with suspension of Krebs-2-carcinoma cells in ascitic fluid.

experiment there were four suvivors at 25° C. and one at 35° C., and on the 22nd and 23rd days the last mice at the high and low temperatures, respectively, died. This experiment was repeated twice in a slightly different manner in which the inoculated animals were held at 25° C. for 3 and 6 days before half were placed at 35° C., and the results are presented as II and III, respectively, in text-figure 1. In these last two experiments the interaction of high temperature and the rapidly growing tumor proved to be much more lethal than when inoculated animals were held at 25° C. for only a few hours before being placed at 35° C. Tumors growing in surviving animals at 35° C., however, gave essentially the same results as in the initial experiment. The absence of an effect of high temperature on the growth of the Krebs-2 carcinoma is completely in line with the *in vitro* metabolic data obtained in this laboratory (*2*). No differences in the ability to utilize glucose as substrate have been found in this tumor from hosts held at 25 and 35° C. (*2*).

S91 Melanoma and S91A Amelanotic Melanoma

Since the S91A is an amelanotic derivative of the S91 melanoma, it was desirable to study the effects of temperature on these tumors concurrently. Twelve strain DBA males were inoculated as previously described with S91A tumor tissue, and others were similarly inoculated with S91 tumor tissue. Both tumors had been carried in DBA mice of the same subline as those used for the experiment. Two cages each contained 6 mice per cage inoculated with S91A, and 2 cages each contained 6 mice and 5 mice, respectively, inoculated with S91. When the tumors were first palpable (after 9 days for the S91 and after 12 days for the S91A), they were measured and half of the animals were placed at 35° C. Text-figure 2 shows the effect of high temperature on the average growth rate of both tumors.

It is apparent from text-figure 2 that the growth patterns of the two tumors are different. In the S91 tumor there was a marked inhibition of growth up to 12 days after exposure to high temperature. Measure-

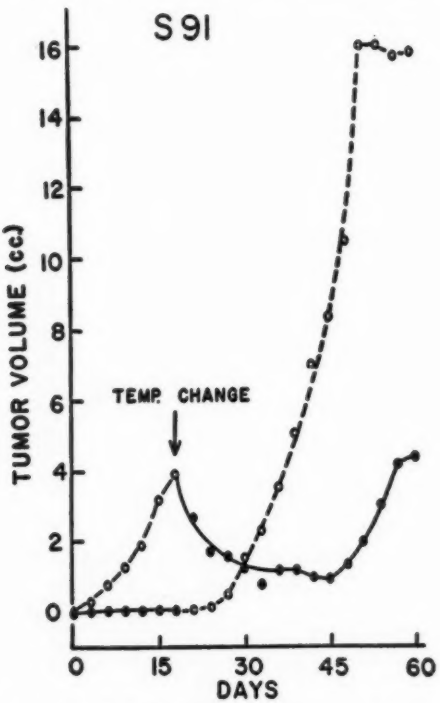


TEXT-FIGURE 2.—Comparative growth rates of the S91 melanoma and the S91A amelanotic melanoma at 35° C. (*solid line*) and 25° C. (*dash line*) expressed as average tumor volume plotted against time.

ments taken on the 12th day of exposure to the controlled temperatures showed an average tumor volume of 0.05 cc. for tumors in animals kept at 35° C. as compared with an average volume of 2.0 cc. for tumors in animals kept at 25° C. On the 21st day, measurements of the S91 tumors held at 35° C. indicated that growth had occurred. These tumors continued to grow, but their average volume remained very much less than the average volume of tumors in animals held at 25° C. There was no significant effect of 12 days' exposure to high temperature on the growth of the S91A tumors. The average tumor volume for S91A tumors in animals held at 25 and 35° C. was 0.6 cc. after 12 days at the controlled temperatures. From that time on the S91A tumors held at 35° C. began to grow more slowly and after a total of 45 days' continued exposure there was a significant difference in average volume between S91A tumors in animals held at 25 and 35° C. The more moderate effect of high environmental temperature on the growth of the S91A as compared to its effect on the growth of the S91 is correlative to the response to insulin and to testosterone *in vitro* by these tumors (*2*).

If the effects on tumor metabolism as measured by *in vitro* metabolic studies and *in vivo* growth studies are indeed the result of a disturbed endocrine balance resulting from stress, the reaction might be expected to be a reversible one. Heat-sensitive tumors grown at 25° C. should show growth inhibition when placed at high temperatures, and conversely the same tumor grown originally at high temperatures should resume growth when placed at low temperatures. To determine whether or not this was indeed the case, 7 mice were inoculated with S91 tumor in the manner previously described and were weighed, numbered, and placed at 25° C. After 6 days at 25° C., 4 mice were placed at 35° C. Growth of the tumors in animals placed at the higher temperature was inhibited, while tumors at the lower temperature grew well. After 15 days' exposure, the mice held at 35° C. were placed at 25° C. and those continuously exposed to 25° C. were placed at 35° C. As shown in text-figure 3, the inhibited tumors in mice held at 35° C. grew at a rate normal for the S91 when the host was placed at 25° C. Conversely, there was a sharp but temporary regression of tumors grown in animals held originally at 25° C. when the hosts were placed at 35° C. The average tumor volume of these animals declined until the tumors were one fourth the volume they had reached at 25° C. From 15 to 27 days after exposure, the average tumor volume remained generally at this level. After 27 days' exposure to high temperature, however, these tumors resumed growth at a rate paralleling that observed at the lower temperature. The average tumor volume reached a peak after 42 days of exposure to 35° C. It would seem that some sort of selection occurred during the period of the plateau (33 to 45 days, in text-fig. 3) that resulted in the ascendancy of a type of S91 melanoma cell that would grow as well at 35 as at 25° C. Autopsy revealed that these tumors were macroscopically lighter in color than tumors continuously inhibited by high temperature and that there were extensive amelanotic areas.

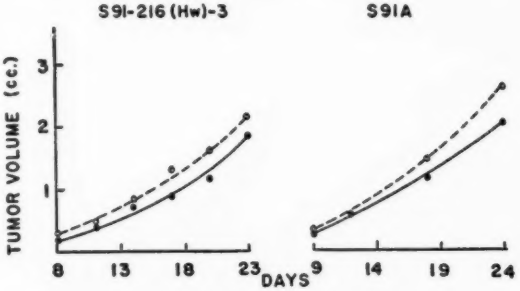
To determine whether these amelanotic foci consisted of tissue which would grow well at 35° C., tissue from these areas was transplanted into other DBA males. After 7 days at 25° C., to allow the mice to recover from the effects of inoculation, they were kept continuously at 35° C. Tumors resulting from these inoculations did indeed grow well at high temperature, and subsequent transplants were made. In later transplant generations in this series of derived tumors [S91-216(Hw)], the mice were inoculated with the most macroscopically amelanotic tissue from the



TEXT-FIGURE 3.—The effect of changing the environmental temperature of the host from 25° C. (*dash line*) to 35° C. (*solid line*) and *vice versa* on the growth of the S91 melanoma. Growth rates expressed as average tumor volume plotted against time.

tumor of the preceding generation which grew best at 35° C. Mice bearing the third transplant generation (S91–216(Hw)-3) were exposed to 35 and 25° C. for 24 days (text-fig. 4), after which time three animals exposed to the higher temperature and two animals exposed to the lower temperature were sacrificed. Tumors from these mice were removed, and their response to exogenous insulin and testosterone in terms of $Q_{CO_2}^{N_2}$ was determined manometrically. The tissue which constituted these tumors was separated into dark- and light-colored portions to see whether or not degree of melanization would have any effect on the $Q_{CO_2}^{N_2}$.

The dark- and light-colored portions of tumor tissue were pooled, and 100 mg. (wet weight) tissue samples of the grossly dark- and the grossly light-colored tissue were used for the experiment. In general, slices from the derived tumors kept at either environmental temperature showed a metabolic pattern similar to that of the S91A, *i.e.*, there was a considerable reduction in percentage inhibition of $Q_{CO_2}^{N_2}$ by testosterone, temperature stress, or both, and a lowered sensitivity to exogenous insulin (*1*, *2*). The data from this experiment are presented in table 1. It is interesting to note that exposure of animals bearing the derived tumor to a lower environmental temperature did not significantly alter *in vitro* reactions of the tumor to exogenous hormones. Since there was no significant manometric difference between the light and dark tissue, one must conclude that the presence or absence of melanin is probably not a factor in the glycolytic responses of these tumors. Further similarity between this metabolically atypical S91 tumor and the S91A is seen in text-figure 4, which presents



TEXT-FIGURE 4.—Comparative growth rates of the derived tumor S91-216(Hw)-3 and the S91A amelanotic melanoma at 35° C. (*solid line*) and 25° C. (*dash line*) expressed as average tumor volume plotted against time.

TABLE 1.—$Q_{CO_2}^{N_2}$ *values obtained with tissue samples of S91-216(Hw)-3 from mice exposed to 35 and 25° C. for 24 days*

Tissue sample	$Q_{CO_2}^{N_2}$ at 20° C.*			$Q_{CO_2}^{N_2}$ at 40° C.*		
S91-216(Hw)-3 at 35° C.	Control	Plus insulin	Plus testosterone	Control	Plus insulin	Plus testosterone
Pooled dark slices	2. 3	2. 2	0. 54	14. 8	14. 6	4. 9
Pooled light slices	2. 4	2. 4	0. 37	14. 4	16. 4	5. 4
S91-216(Hw)-3 at 25° C.						
Pooled dark slices	1. 6	1. 9	0. 88	10. 2	11. 8	5. 0
Pooled light slices	1. 8	2. 1	0. 90	10. 0	10. 4	5. 0

*$Q_{CO_2}^{N_2}$ based upon μl. CO_2 mg. dry wt. per hr. Krebs-Ringer's, 0.04 *M* $KHCO_3$, 0.625 percent dextrose (5 ml. per vessel); crystalline zinc insulin (Lilly Lot No. 535664) 3.9 mg. per 10 ml. H_2O (0.2 ml. per vessel); testosterone (Schering) 9.6 mg. per 2 ml. ethyl alcohol (absol.) plus 10 ml. water (0.5 ml. per vessel). Slices were incubated in the Warburg bath consecutively at 20 and 40° C.

the average growth rate of the S91-216(Hw)-3 compared to that of the S91A for similar periods of exposure to 25 and 35° C.

Discussion

Recent work has shown that an environmental temperature of 35° C. inhibits the growth of S91 melanoma in strain DBA mice and that this inhibition is concomitant with a lowering of $Q_{CO_2}^{N_2}$ (*1*). This decrease in anaerobic acid production is intensified by exogenous testosterone and is insulin-reversible (*1, 2*). The data here reported indicate that there is a differential growth response of three transplanted tumors in mice kept at an environmental temperature of 35° C. This response ranges from no growth inhibition in the Krebs-2 carcinoma through moderate inhibition in the S91A amelanotic melanoma to marked inhibition in the S91 melanoma. The fact that some tumors grow at 35° C. suggests that growth inhibition of tumors at high temperature is not a physical phenomenon, *e.g.*, dehydration. Rather, this effect is more probably the differential response of these tumors to host endocrine changes in response to a stressing agent, namely, high temperature (*2, 5*). Selye (*5*) and Bullough (*14*) have pointed out that any change from the optimum in an animal's environment can be regarded as a stressing agent if it induces attempts at resistance. Herrington (*15*) has shown that an environmental temperature of 31.5° C. is the optimum for mice when normal activity is allowed. Our observations of tumor-bearing animals indicate that exposure to 6.5° C. below the optimum temperature does not affect tumor growth, but that exposure to 3.5° C. above the optimum temperature results in growth inhibition in certain tumors. However, high temperature is not the only stressor that triggers host responses which result in tumor inhibition. Rashkis (*16*) found that stressing Swiss albino mice by forced swimming inhibited the growth of an unnamed ascites tumor and of a sarcoma induced by injection of methylcholanthrene.

Although there is some inhibitory effect of high environmental temperature on the growth of Krebs 2, this effect may well be some manifestation of the general adaptation syndrome which does not involve the glycolytic mechanism of the tumor and is therefore not affected by the anti-insulin inhibitor mechanism described elsewhere (*2*). The higher rate of mortality of Krebs-2-bearing animals held at 35° C. may possibly be the result of the overwhelming combined stress of a rapidly growing tumor and the high environmental temperature. Mice (DBA males) inoculated intraperitoneally with 0.3 ml. of the Krebs-2 carcinoma in ascites form showed an even higher mortality rate, *i.e.*, 5 of 10 animals inoculated and placed at 35° C. 7 hours after inoculation died within 5 days (unpublished).

The present results with S91 are in contrast with those reported previously (*1*) where only very little growth occurred during an exposure to 35° C. for 39 days. It was observed that in those S91 tumors which had begun to grow at 35° C. after a prolonged quiescence, there were extensive areas of amelanotic tissue. Cytologically, these tumors revealed patterns of cellular melanization similar to those already reported as occurring in

irregularly melanized S91 tumors grown at ambient temperature (*17*, *18*).

Grossly amelanotic tissue from these tumors transplanted into other strain DBA male mice grew well at 35° C. After 3 transplant generations of derived S91 tumor tissue from animals held at 35° C. and selected for amelanosis from the fastest growing tumor in each preceding transplant generation, the growth rate and *in vitro* metabolic response of the tumors from the third transplant generation to exogenous insulin and testosterone were quite similar to the situation in S91A. Subsequent transplant generations of the S91–216(Hw) tumor (up to the eighth), however, continue to contain a mixture of amelanotic and melanized cell types, with the balance in favor of cells with colorless or lightly melanized granules [mitochondria (*17–19*)]. Grossly amelanotic tissue was selected for transplantation because in the S91 melanoma and its derivatives there appeared to be some connection between loss of the ability to produce melanin, ability to grow well at 35° C., and relative insensitivity to exogenous insulin and testosterone *in vitro*. This seems to be true in spite of the fact that tissue from these tumors reacted uniformly to exogenous hormones *in vitro* regardless of degree of melanization. S91 tumors which failed to show appreciable growth in mice maintained at 35 to 40° C. for 10 to 44 days were uniformly very dark in color and highly sensitive (*1*, *2*) to exogenous insulin and testosterone.

The S91 melanoma has been observed to contain tumor cells of three types with respect to cytoplasmic granules: cells with melanized granules only, cells with colorless granules only, and cells with varying proportions of melanized and colorless granules (*17*, *20*). The data presented indicate that in the S91 melanoma growing in an animal maintained at 35° C., growth of the amelanotic cell type is favored.

Summary

A differential growth response of three transplanted tumors in strain DBA mice kept at environmental temperatures of 35 and 25° C. is reported. The responses at 35° C. range from slight growth inhibition in the Krebs-2 carcinoma through moderate inhibition in the S91A amelanotic melanoma to marked inhibition in the S91 melanoma. This effect reflects in part a differential response of these tumors to possible endocrine changes in the host in response to a stressing agent, namely, elevated temperature. In the S91 melanoma, where growth is sharply inhibited when the host is placed at 35° C., this inhibition is reversed when the host is placed at 25° C. Exposure to 35° C. is detrimental to animals bearing the Krebs-2 carcinoma but not to those having the S91 or S91A. Data presented indicate that in the S91 melanoma growing in mice maintained at 35° C., growth of the amelanotic cell type is favored.

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A Phase-Contrast Microscope Study of Free Cells Native to the Peritoneal Fluid of DBA/2 Mice[1]

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Using the phase-contrast microscope, a study has been made of the morphology of the free cells indigenous to the peritoneal fluid of strain DBA/2 mice under control and experimental conditions. In recent years there has been an expanding volume of literature involving the use of tumor cells growing as free cells in the peritoneal fluid of experimental animals. Although some of these reports have concerned themselves in part with the cells native to the peritoneal fluid, the area of concern has been chiefly with the response of the various cell types to tumor growth or to tumor-affecting conditions. A detailed morphologic study of these cells alone has not appeared. It was felt that, in the light of the frequent need for accurately distinguishing between neoplastic and non-neoplastic cells, further analysis was justified.

There exists a large body of basic information on the free cells of the serous fluids—the result of careful and extensive research carried out by a number of investigators during the past 50 years, notably Pappenheim and Fukushi (*1*), Cunningham (*2*), and Maximow (*3*). Staining techniques and the light microscope have been used in these studies as well as in most of the present-day work on experimental free-cell tumors and in that involving the identification of tumor cells in human serous effusions. Since the phase-contrast microscope makes it possible to study the cells from the peritoneal fluid in the living state under conditions in which both nuclear and cytoplasmic detail are visible, it was chosen in preference to classical or present-day staining methods.

Materials and Methods

The mice used for this study were 2- to 3-month-old strain DBA/2 mice of both sexes. They were fed Purina laboratory pellets *ad libitum*. The peritoneal fluid from these animals was the source of the cells for both control and experimental studies.

Peritoneal fluid was collected from the mice by means of abdominal puncture and the use of a capillary pipette for aspiration. Immediately

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after aspiration, coverslip preparations were made. The preparations were sealed with a mixture of 65 percent paraffin and 35 percent balsam. These preparations were used for determining the proportions of the various cell types.

The sources of peritoneal fluid were as follows:

1) From control mice, mentioned above.
2) From mice injected intraperitoneally with 1 cc. of 0.85 percent sodium chloride daily for 3 days.
3) From mice inoculated intraperitoneally with ½ cc. of a suspension of melanin granules obtained from melanoma S91 implanted on the peritoneal wall. Approximately 3 grams of the tumor was put through a tissue press and suspended in distilled water to make a volume of about 25 cc. This was placed in a heavy centrifuge tube and beaten with a stainless steel blade for 10 minutes using a Dumore motor set to run at 90 volts (approximately 8,000 r.p.m.) in order to break up the cells. The large particles were thrown down by centrifuging at 1,500 r.p.m. for 5 minutes. The supernatant, containing the melanin granules, was centrifuged for 10 minutes at 40,000 r.p.m., and the sediment was resuspended in distilled water and again centrifuged for 10 minutes at 40,000 r.p.m. The resulting residue was resuspended in 0.85 percent NaCl and used for the inoculation. The size of these granules was about 0.2μ or less, and they were smooth at the magnification of the phase-contrast microscope.
4) From mice injected intraperitoneally with 1 cc. of India ink diluted 1:15 with 0.85 percent NaCl. The diluted ink was heated to boiling in a water bath for 20 minutes and cooled before inoculation. The suspended carbon particles were for the most part larger than the melanin granules, and many were sharp and jagged in outline.

In some cases smears of the peritoneal fluid cells were air-dried and stained with Wright's stain. Wet smears were fixed in Champy's fluid and stained with the Bensley-Cowdry acid fuchsin, methyl green stain for mitochondria.

The microscope and camera used was a Leitz Panphot with Heine condenser and Bausch and Lomb oil-immersion phase-contrast objective. The light source was a 100-watt Mikrark illuminator.

Results

A. Staining Techniques for Cells of the Peritoneal Fluid

Smears of cells of the peritoneal fluid stained with Wright's blood stain were found to be useful as a reference in the earlier phases of this study. Identification of cell types stained with Wright's stain depends chiefly upon color differences and gross cell form, little detail being evident in either the cytoplasm or the nucleus. However, the well-established criteria within their acknowledged limitations were of some primary assistance. Also preparations of this type served as a semipermanent record in some of the experimental phases of this study.

The Bensley-Cowdry acid fuchsin, methyl green stain gave a closer approximation to the living state as visualized with the phase-contrast microscope, since mitochondria were stained and much greater nuclear detail was visualized than with Wright's stain. However, the staining procedure is relatively long, complicated, and capricious, and the preparations were not long lasting. Therefore, as the study progressed and greater familiarity was established with the various cell types with phase-contrast, photomicrographs were found to be a more satisfactory permanent record, and the staining methods were for the most part discontinued.

B. Phase-Contrast Microscopy of Cells of the Peritoneal Fluid

As indicated in the section on Materials and Methods, cells of the peritoneal fluid were studied in sealed coverslip preparations. In order to visualize clearly both nuclear and cytoplasmic detail, it was found necessary to subject the cells to some degree of coverslip pressure. In the large majority of preparations the cells made a rapid adjustment to this condition and were found to extend pseudopodia and to show evidence of movement as long as 24 hours after the preparations were made. Since the depth of focus of the oil-immersion lens was small, it was found necessary to photograph the cells shortly after their isolation in order to demonstrate nuclear and cytoplasmic detail in one focal plane. This was impossible after the extension of pseudopodia. In some instances (for example, the large macrophages containing ingested material) this pressure was of such a degree that the cytoplasmic edges were distorted, but it was felt that the integrity of the cytoplasmic and nuclear detail was nevertheless preserved.

1. The Cell Population of the Peritoneal Fluid of Control Mice

Macrophages.—Macrophages composed from 30 to 45 percent of the cells in the peritoneal fluid of control mice and were among the largest cells present (fig. 1). The nucleus was round, oval, or kidney-shaped, with a thin but well-defined nuclear membrane which had occasional areas of apparent variation in thickness. The chromatin was in the form of small, irregular masses and fine strands which possessed relatively low contrast and were scattered throughout the nucleus. The nucleoli numbered 2 to 5 and were small.

The cytoplasmic outline was irregular rather than smooth. When the cell was spherical and the preparation was thick enough so that there was no distorting pressure from the coverslip, the edges were jagged or sharply wavy with occasional or many tiny spicules. These spicules must not be confused with the numerous long, thin, spike-like processes which appear in most cell types under adverse conditions. The cytoplasm had an opaque quality and contained numerous mitochondria, which varied in form from spheres to slender rods and filaments. Many of these cells contained one to several highly refractile droplets, even when there was no evidence of phagocytic activity. Several vacuoles were always in evidence in the inactive macrophage and a few dark-appearing granules

could often be seen. In many macrophages, depending upon the angle at which they happened to be viewed, the cytocenter and the centrosomes could be visualized, occasionally very clearly (see figs. 7, 10, and 15). The nucleocytoplasmic ratio was relatively low.

Lymphocytes.—The lymphocytes of the peritoneal fluid appeared much the same as typical lymphocytes of the blood and other tissues. They composed from 50 to 65 percent of the cell population in control mice. Small and large types of this cell could be found, the small one being the smallest cellular component of the fluid (figs. 1 and 7).

The nucleus was round, slightly indented or lobed, and the nuclear membrane was heavy and appeared to be of uneven thickness. Chromatin was distributed in coarse strands and clumps throughout, appearing darker for the most part than the chromatin strands in macrophages. Occasionally a small nucleolus could be seen. Large lymphocytes had slightly larger nuclei with less dense chromatin.

Lymphocytes had a small amount of translucent cytoplasm which possessed a smooth outline. A few coarse, rodlike mitochondria were present. The large lymphocyte had more cytoplasm and the mitochondria were more numerous than in the small lymphocyte. There was an occasional granule, appearing dark under the phase-contrast microscope, in the cytoplasm of both types.

Mesothelial cells.—In peritoneal fluid from control mice mesothelial cells composed a very small percentage of the cells present—0.5 to 2 percent. There was very rarely a concentration as high as 5 percent. They were chiefly enspherulated and dying cells which apparently had sloughed off from the mesothelium (figs. 1 and 2). Occasionally single cells or sheets of several cells in their squamous form were seen which appeared to be in good condition, but these were most often found in this state after repeated puncture of the peritoneal wall or other irritation (figs. 3 and 8).

The nucleus was round or oval and, in the squamous form, the nuclear membrane was sometimes seen to be folded (fig. 8). It was about the same size as the nuclei of many macrophages but with an ill-defined nuclear membrane. The chromatin was in fine strands of very low contrast and more evenly distributed than in the nuclei of macrophages. There were several prominent but small nucleoli to be seen at one focal level.

The cytoplasm of the dying, enspherulated mesothelial cell was blebbed and irregular (figs. 1 and 2). These cells were very delicate and disintegrated rapidly, even when all other cells showed no adverse effects of an unnatural environment. The quality of the cytoplasm in the squamous form was netlike and an occasional bleb sometimes appeared on the edges, as in figure 8. It was difficult to distinguish the mitochondria but most of them appeared to be tiny, short rodlike bodies. There were many evenly distributed, small, round, dark granules scattered throughout the cytoplasm which varied but little in size, giving the impression of uniformity. These granules were more obvious in the squamous form

than in the disintegrating cells. An occasional small refractile droplet could be seen in both forms. In the squamous cells the cytoplasmic area was large in relation to the nucleus, whereas in the enspherulated, blebbed, and dying cells this relationship was less obvious. In such cells the nuclei appeared larger, possibly as a result of swelling (compare figs. 2 and 3).

Neutrophilic granulocytes.—Very few, if any, neutrophilic granulocytes were to be found in the peritoneal fluid of control mice (0–2%). Because of its continuous and rapid motility it was a difficult cell to photograph, or even to see in detail. Since the best photographic examples of this cell type in the present study were obtained from mice in which conditions of stimulation were initiated, these are the only illustrations presented (fig. 4 and see figs. 10 and 17).

The nucleus of the neutrophilic granulocyte was elongated and lobed, very much as in classical stained preparations with, perhaps, not such obvious and thin constrictions between the lobes. When the ends overlapped or made contact, the characteristic doughnut shape of the rodent granulocyte resulted. The chromatin, though low in contrast, was arranged in large discrete masses. Nucleoli could not be identified. In this cell type the nucleocytoplasmic ratio was relatively high.

The cytoplasm was usually smoothly wavy in outline. It was packed with minute granules which could be seen upon careful focusing and which were probably the neutrophilic granules found in classical stained preparations. They are not visible in the photographs. It was not possible to visualize the mitochondria because of the granules and the movement. The neutrophilic granulocytes illustrated in figures 4, 10, and 17 all contain phagocytosed material.

Eosinophilic granulocytes.—Eosinophilic granulocytes were always present in control-mouse peritoneal fluids in a concentration of from 1 to 5 percent. These cells presented a typical appearance under phase contrast (fig. 5). Their size was about that of the neutrophilic granulocytes but their movement was slower and more sluggish. The nucleus was often doughnut-shaped or like a figure eight and apparently similar in structure to that of the neutrophilic granulocytes. The cytoplasm was full of relatively thick, rodlike granules which were the characteristic acidophilic granules seen in stained preparations. In figure 5 the cell is stationary and the granules are clearly visualized. In figure 1 two eosinophilic granulocytes may be seen, but here there is greater depth and more movement so that the details are not so clear.

Mast cells.—Mast cells, with the characteristic morphology of those found in connective tissue, were always present in control-mouse peritoneal fluid in a concentration of from 0.1 to 1 percent. These cells were larger than the neutrophilic and eosinophilic granulocytes, with more cytoplasmic area in relation to the nucleus (fig. 6). The nucleus was round or kidney-shaped. Its detail was difficult, if not impossible, to see because of the specific granules which were large and which completely filled the cytoplasmic area. These granules were gray under phase contrast.

2. *Cellular Reaction to Stimulation*

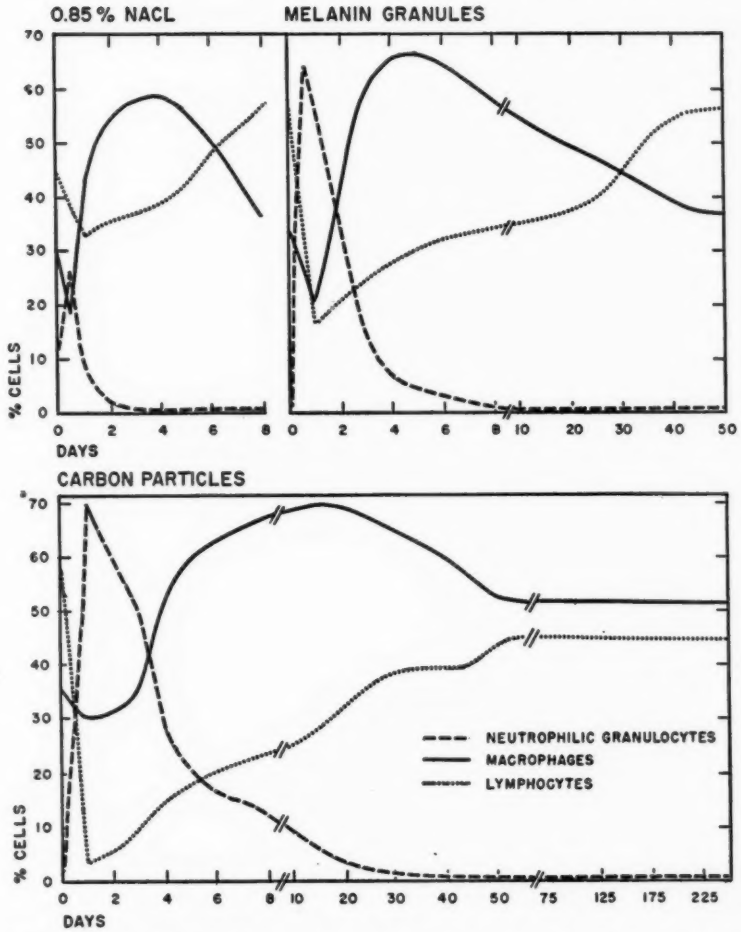
Of the three types of stimuli used, 0.85 percent sodium chloride served as a mild irritant, melanin granules as an example of native particulate matter, and carbon particles in India ink as a source of inert particulate matter. Text-figure 1 shows, in graphic form, a comparison of the relative proportions of the three main cell types reacting to these three stimuli: neutrophilic granulocytes, macrophages, and lymphocytes. For the purpose of this comparison, the saline reaction is shown from just prior to the last of three inoculations.

Sodium chloride (0.85%).—Three daily intraperitoneal injections of 1 cc. each were given to a group of strain DBA/2 mice. The first injection caused only a slight rise of neutrophilic granulocytes. Both the second and third injections caused a marked rise in these same cells, to as high as 27 percent in 6 hours. Each rise had sharply receded in the 18 hours preceding the next inoculation. Forty-eight hours after the third and last injection they had returned to approximately the control level. After the third inoculation the macrophages and lymphocytes showed a mild and transitory reaction and by the eighth day had approached control levels. The form of the curve of their relative proportions was similar to that resulting from the more prolonged reactions to stimulation with melanin and carbon particles (text-fig. 1).

The chief changes in the macrophages following injection of saline was the appearance of larger numbers of refractile droplets and small granules which appear dark under phase contrast (fig. 7). Occasionally cell debris or an ingested cell were seen (fig. 8). Numerous transitional forms between lymphocytes and macrophages became an outstanding part of the cell picture after the granulocytic reaction subsided (fig. 7). Some of these cells were the size of large lymphocytes, while others were comparable in size to small macrophages. The nuclei were oval or elongated, bent ovals. The nuclear membranes appeared thinner and less uneven than in the lymphocytes. The distribution of the chromatin was more diffuse and the chromatin masses were less dense than in the lymphocytes, whereas nucleoli, when seen, were larger than those of the lymphocyte. The cytoplasm had some of the translucent quality of the lymphocyte, but the cytoplasmic edges were less smooth and mitochondria were finer and more numerous. Fewer vacuoles were apparent in the cytoplasm than in the mature macrophage; however occasional characteristic refractile droplets and dark granules were present. The cytocenter was visible in some cells which still possessed many of the characteristics of lymphocytes (fig. 7).

Macrophages in mitosis were occasionally found in the peritoneal fluid of control mice. Under any condition of stimulation, such as repeated daily puncture of the peritoneum, there was an increase in their occurrence —occasionally as high as 1 percent. Figure 9 shows a macrophage in metaphase which is illustrative of those seen after inoculation with saline and other kinds of stimulation.

In the 24-hour period following the first injection of saline there was a



TEXT-FIGURE 1.—The proportions of three cell types native to the peritoneal fluid of strain DBA/2 mice during the period of response to three types of intraperitoneal stimulation.

marked increase in mesothelial cells (rarely as high as 10 percent), many in their squamous form (fig. 8). There was no increase over control proportions during the remainder of the test period.

Before the inoculation of saline, the eosinophilic granulocytes averaged 3 percent. Throughout the period after the inoculations they remained for the most part on the high side of control proportions. Mast cells remained at control levels throughout the test period.

Melanin granules.—DBA/2 mice inoculated intraperitoneally with ½ cc. of a suspension of melanin granules in 0.85 percent sodium chloride showed a more intense and more prolonged reaction on the part of the three reacting cell types than in the case of the reaction to saline (text-fig.

1). For the first few days there were free melanin particles present in the peritoneal fluid (figs. 10 and 11). These free particles slowly diminished in number, and by the 9th to the 13th day they had disappeared. At autopsy, 108 days after inoculation, macroscopically visible melanin deposits could be seen in the omental area.

Ingestion of melanin granules by free cells was evident from the first samplings at 18 and 24 hours. At this time the reaction of the neutrophilic granulocytes was at its height, and scattered, discrete melanin granules could be seen in many of them (fig. 10). Small macrophages had begun to ingest the particles, which were frequently found to be distributed in clumps, chiefly around the area of the centrosome (*arrow*, fig. 10). Situated in the cytoplasm of other macrophages were vacuoles of varying sizes, 1 to 4μ, containing closely packed melanin granules (fig. 11). By 48 hours there were cells containing very large numbers of the melanin-filled vacuoles (fig. 12). An increasing number of large, engorged macrophages appeared having closely packed melanin-containing vacuoles measuring up to 8μ in diameter. From about the third day on enormous macrophages were found with two types of cytoplasmic vacuoles, one containing closely packed melanin granules, the other (of a similar size range) containing but few melanin particles. The particles in these latter vacuoles (fig. 13) were in Brownian movement and therefore not clearly resolved. Some cells possessed vacuoles of both types, while in others but one type of vacuole was present (figs. 12, 14). Many of the cells with vacuoles containing loosely packed granules were of great size and contained numerous large refractile droplets which gave them a brilliant and luminous quality (figs. 13, 14). These refractile droplets, which seemed to increase to some extent under any condition of stimulation, varied from a fraction of a micron to over one micron in diameter. The very large macrophages with both types of vacuoles reached a peak on the fifth day when they comprised the majority of this cell type. From then on they declined in relation to the total number of macrophages.

Throughout the intense phase of the cellular reaction there was a great variation in the size of the macrophages and they exhibited a marked pleomorphism. Nucleoli varied in size and the nucleocytoplasmic ratio was not constant (figs. 7, 8, and 10 through 15). In figure 15 the smallest cell in the clump is a transition form, containing a few discrete, ingested melanin granules. Figure 16 shows a macrophage in late anaphase with a number of melanin-containing vacuoles in the cytoplasm. Macrophages showed a tendency to clump, even in control fluids (fig. 1). In animals in which some kind of stimulation had been initiated the clumps were very obvious (figs. 11, 14, and 15). They may be composed of a large number of cells. Sometimes a few lymphocytes are incorporated in the clump or grouped around the edges, but the main mass of cells is composed of macrophages.

The macrophages, after reaching their peak at 5 days, were predominant over other cell types for about 30 days. During all this period varying amounts of melanin were found in most of them. Many melanin-contain-

ing macrophages were seen through 60 days, and occasionally one was seen after 100 days.

On the second day of the experiment the largest number of mesothelial cells appeared (3%). The great majority were of the squamous type and in a fairly well-preserved state (fig. 3). Except for this period the mesothelial cells ranged from 0 to 2 percent.

Eosinophilic granulocytes remained on the low side of control proportions throughout. Mast cells, while mostly below 0.1 percent were invariably seen during the first 9 to 14 days and thereafter reassumed their control level.

Carbon particles from India ink.—After the intraperitoneal inoculation of 1 cc. of a 1:15 dilution of India ink in 0.85 percent sodium chloride, the picture of the reaction of the three chief responding cell types was mainly one of greater intensity and longer duration than after the inoculation of melanin granules (text-fig. 1). Until the end of the test period of 250 days, macrophages were still predominant. At autopsy, on 50 and 250 days respectively, black deposits of carbon were macroscopically visible on the omentum, at the point of inoculation, and scattered throughout the mesentery. Small amounts were also present on the diaphragm and the abdominal wall.

Particles were found to be free in the peritoneal fluid for the first 9 to 13 days. Directly after inoculation all the mice became ruffled, sluggish, and unresponsive. This condition lasted for several days, with but slow improvement. However, by 7 days they again appeared to be normal.

At the beginning, carbon particles were ingested by the neutrophilic granulocytes, some of them containing considerable amounts (fig. 17). A number of refractile droplets and vacuoles were also present. Many disintegrating granulocytes were seen during the early stages of the experiment.

Macrophages also immediately started ingesting the carbon particles. In the first 24 to 48 hours many containing a number of large vacuoles (up to 5μ in diameter) were seen in the cytoplasm (fig. 18). In these vacuoles varying numbers of large, irregular particles or clumps of smaller particles could be seen, filling but a small portion of the vacuole. Other macrophages were of the type characteristic of the early hours of ingestion of melanin granules and contained no vacuoles. In the following several days fewer of the large, partially filled vacuoles were seen. Larger macrophages containing increased amounts of carbon (fig. 19) began to appear. Groups of these particles were closely packed in vacuoles as were the melanin granules (see fig. 12). However, due to the asymmetry of the carbon particles, the vacuoles under these conditions never appeared round, and their size at the greatest was 4 to 5μ, considerably less than the largest of the melanin-packed vacuoles.

There was an increase in refractile droplets from the start of the experiment in the neutrophilic granulocytes and macrophages, being more marked in the granulocytes under these conditions than during the reaction to melanin granules. At no time did the number of refractile

droplets increase in the macrophages to the extent to which they increased after the injection of melanin granules. There was the impression of more frequent cell death among the macrophages which had ingested carbon particles than among those which had ingested melanin granules.

Large macrophages began to appear with greater frequency after the third day and were dominant from about the 10th to the 23rd days, but they never attained the enormous size resulting from inoculation with melanin granules. Macrophages engorged with carbon particles were found often during the first 7 to 10 days and with marked decreasing frequency thereafter. However the majority of macrophages continued to contain varying amounts of carbon throughout the test period of 250 days (fig. 20).

Numerous cells in transition between lymphocytes and macrophages were a prominent part of the picture for 13 to 17 days and a more minor but continuing part for over 100 days. Some of them were seen to contain a few ingested particles of carbon. Macrophages in mitosis were also found.

Mesothelial cells conformed, on the whole, to the picture found with melanin granules. Occasionally one was seen, either in the squamous form or enspherulated and moribund, containing a few discrete particles of carbon.

Eosinophilic granulocytes were on the low side of the control level in relative proportion throughout the test period. No mast cells were observed until about 40 days after inoculation. Later they were seen, but very rarely, and only when thousands of cells were scanned. Toward the latter part of the test period their numbers approximated control levels.

Discussion

Thirty years ago Cunningham (*2*), in his excellent analysis and review of previous work, stated that, of the free cells of the mammal, with the exception of the free cells of the circulating blood, the normal cells of the serous cavities have probably been the most extensively studied. However, the major portion of this work has been done on experimental animals, the paper by Dixon and Rixford (*4*) containing the only description we could find of the cell population in normal human peritoneal fluid. Although this study involved relatively few cases, analogy with the extensive knowledge of the cells of the peritoneal fluid of animals allows the conclusion that the normal human peritoneal fluid contains the same range and similar proportions of cell types as in laboratory animals and that its cellular response to stimulation follows a like course.

Cell types and proportions have been found to be similar in the fluids of all the serous cavities (*5*). In addition, in the study by Key (*6*) on the cytology of the synovial fluid of children and laboratory animals, similar cell types and proportions were found. The peritoneal fluid of laboratory animals is most convenient for detailed work and has been widely used for basic studies and experimental purposes.

Certain facts concerning the free-cell population of the peritoneal fluid

of mammals in general have been well established. The milky spots, or the densely cellular areas of the omentum, as well as the submesothelial tissue in general, have been shown to be the chief source of the free cells (*1–3, 5, 7, 8*). In these areas all the cell types of loose connective tissue are found. The milky spots are macroscopically visible in some species, especially in the rabbit and man (*8*). Similar densely cellular areas can be found in the serous membranes of the pleural cavities (*5*). The majority of the macrophages which appear under inflammatory stress come from these areas and few, if any, from the blood stream. They may attain enormous size and often tend to float in clumps in the peritoneal fluid (figs. 11 and 15). Great activity is shown by the macrophages and at times a considerable amount of mitosis is seen. Neutrophilic granulocytes appear from the blood in large numbers only as the initial response to irritation. Their occasional presence in control fluids could be attributed to minute amounts of blood appearing as a result of the abdominal puncture or as the response to a mild inflammatory or some other condition existing in the mouse. In our material, desquamated mesothelial cells occurred in very small numbers in control fluids, were usually dead or dying, and are thus considered to be the result of the normal process of cell replacement. There is general agreement among the investigators mentioned that mesothelial or serosal-lining cells respond only in a mild or passive way to stimulation or irritation and their relative numbers never become large. Lymphocytes, which compose a relatively large proportion of the cell population, are probably derived both from peritoneal tissues and the blood stream. Following stimulation they are considered to be a source of macrophages through their ability to transform into these cells.

Many means have been used to initiate a mild inflammatory reaction in the peritoneal cavity, from egg albumen to partial hepatectomy. The growth of tumor cells also produces a reaction from the native cells and this has been studied by Yoshida (*9*), Goldie and Felix (*10*), and Klein (*11*), among others. The cellular response to these inflammatory reactions has been found to be of the same general form (*9–12*). An initial and immediate invasion by neutrophilic granulocytes, as early as 1 to 2 hours, is followed in 24 to 36 hours by a slow but continuous rise in the numbers of macrophages. The granulocytes begin to recede in 24 hours and slowly return to low or control levels. The speed of this return varies with the substance used and the intensity of the reaction produced. In our hands, foreign particulate matter (India ink) produced a longer and more intense reaction than native particulate matter (melanin granules). Seeley *et al.* (*12*) found a corresponding difference in intensity between amniotic fluid and sodium ricinoleate.

In our study large numbers of cells in transition between lymphocytes and macrophages were always found, especially after the injection of particulate matter which, in both instances, produced more intense and prolonged responses than the use of saline (figs. 7 and 16). These cells closely followed in form the descriptions of Bloom (*13, 14*) in his studies

of the transition of lymphocytes through macrophages to fibroblasts in tissue culture.

Webb (*15*) studied the omentum as well as the free cells in the peritoneal fluid of rats before and after the inoculation of irritating substances: egg albumen, blood charcoal, potassium ferrocyanide, and iron and ammonium citrate. He concluded that in both places the mast cell disappeared. Yoshida (*9*), Goldie and Felix (*10*), and Klein (*11*) noted that following the inoculation of rapidly dividing neoplastic cells into the peritoneal cavity, mast cells were not observed after the first day. In our experiment with saline, mast cells were presented in approximately control proportions throughout the test period. With melanin granules they were below control proportions but always present during the first 9 to 14 days of the experiment and thereafter reassumed their former level. After the inoculation of India ink, no mast cells were seen for 40 days. After this period they were observed but in numbers well below control proportions, until after 125 days when they were found at approximately control levels. Further experiments would be needed to determine whether, after the use of certain substances and after the initiation of tumor-cell growth, the mast cells do totally disappear or whether, in some instances, the absolute increase in cell numbers due to the inoculation of irritating substances or the growth of tumor cells masks their presence.

Most of the earlier studies, as well as much of the more recent work on serious effusions and tumor cells growing as free cells in the serous fluids, have been made using various staining techniques. Many of the important contributions by Pappenheim and Fukushi (*1*), Cunningham (*2*), Maximow (*3*), Bloom (*13*, *14*) and others were done by means of intravital and supravital staining. The use of vital staining has done much to establish the source and migration of various cell types. However, the advantages of being able to study living, untreated cells with the phase-contrast microscope, which gives both nuclear and cytoplasmic detail, are obvious. In our experience it was found that its use enabled us to interpret stained material from the same source more objectively, and details in fixed and stained cells which went unnoticed or were misinterpreted before could be seen or understood.

Before the phase-contrast microscope was introduced into this country in 1946, Albertini (*16*) proposed its use as a new technique for pathologic study, particularly of tumor cells from tissue and from exudates. He reported having studied over 50 exudates and having found its use superior to either stained smears or embedded and sectioned centrifuged pellets. Today the phase-contrast microscope is applied in this area and its use has extended to many other fields as evidenced in the review by Richards (*17*).

With the large numbers of papers appearing in recent years dealing with the growth or presence of tumor cells in serous fluids, there has been no review, to our knowledge, of the previous studies on native cell components or any serious attempt to amplify the basic knowledge in this area. This is true not only in the field of experimental free-cell tumors in the peritoneal

cavity, but in the field of human "exfoliated" cells in serous fluids as a means of early cancer detection. This type of study assumes a knowledge of the normal cell population of the human serous fluids and their response to conditions of stimulation. Not only has the paper of Dixon and Rixford (*4*) establishing the analogy between the human and other mammalian free cells been rarely, if at all, referred to, but also the additions to and knowledge of the cytology of the human fluids contributed by Ivers and Pomerat (*18*) and Coman (*19*) have been largely ignored.

It seemed important to us to point out that this large volume of basic data is available and to demonstrate the value of the phase-contrast microscope in contributing to its further increase. It is suggested that a careful and conscientious review of the basic information plus the use of the phase-contrast microscope as an adjunct in further studies would contribute to a more accurate identification of the variety of non-neoplastic cells in mammalian serous exudates.

Summary

1) Native cells of the peritoneal fluid of strain DBA/2 mice have been studied with the phase-contrast microscope.

2) Cell types present in the peritoneal fluid have been determined in terms of their relative proportions under control and experimental conditions and the details of their cytologic structure have been described.

3) The cellular reaction to stimuli primarily involved the neutrophilic granulocytes, the macrophages, and the lymphocytes. Mesothelial cells appeared to play a passive and minor role.

4) The value of phase-contrast microscopy as an aid to the cytologic characterization of living, unstained cells in serous fluids has been discussed.

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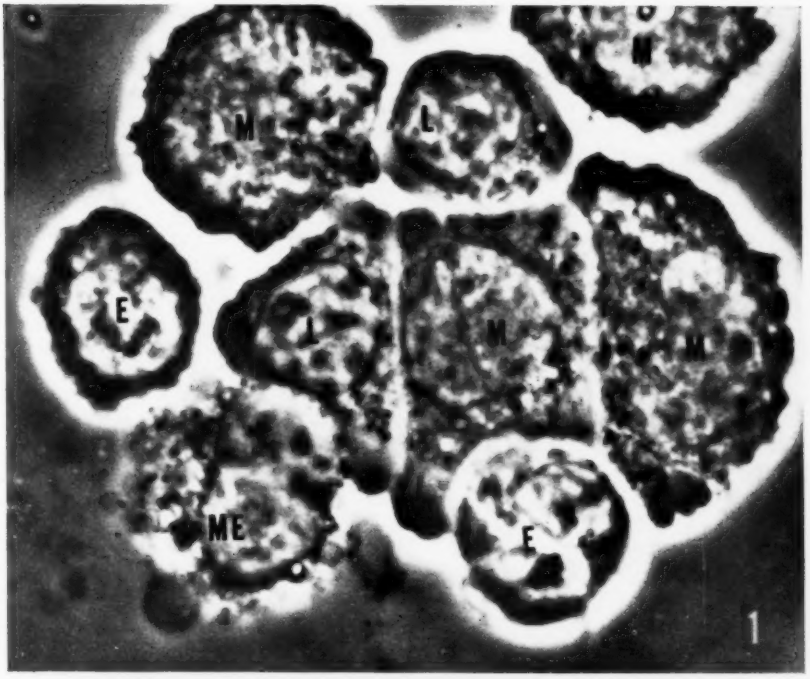
Description of Figures

All figures are phase-contrast photomicrographs of living cells from the peritoneal fluid of strain DBA/2 mice at a magnification of 2,000 ×. All the cells are more or less flattened between cover glass and slide in order to show morphologic detail. When there is gross distortion of the cytoplasmic edges caused by this pressure, mention will be made in the figure descriptions. Unless otherwise stated, labeling will be as follows: B—mast cell; E—eosinophilic granulocyte; L—lymphocyte; M—macrophage; ME—mesothelial cell; N—nucleus; P—neutrophilic granulocyte; T—cell in transition from lymphocyte to macrophage.

PLATE 18

FIGURE 1.—Group of cells from a control mouse. Macrophages, lymphocytes (*upper*, small lymphocyte; *lower*, large lymphocyte), a desquamated, dying mesothelial cell and two eosinophilic granulocytes, the one at the *left* showing the characteristic ring-shaped nucleus.

FIGURE 2.—To the *right* an enspherulated, dying mesothelial cell from a mouse 35 days after the intraperitoneal inoculation of melanoma S91. To the *left* is an unidentified cell exhibiting tripolar mitosis.



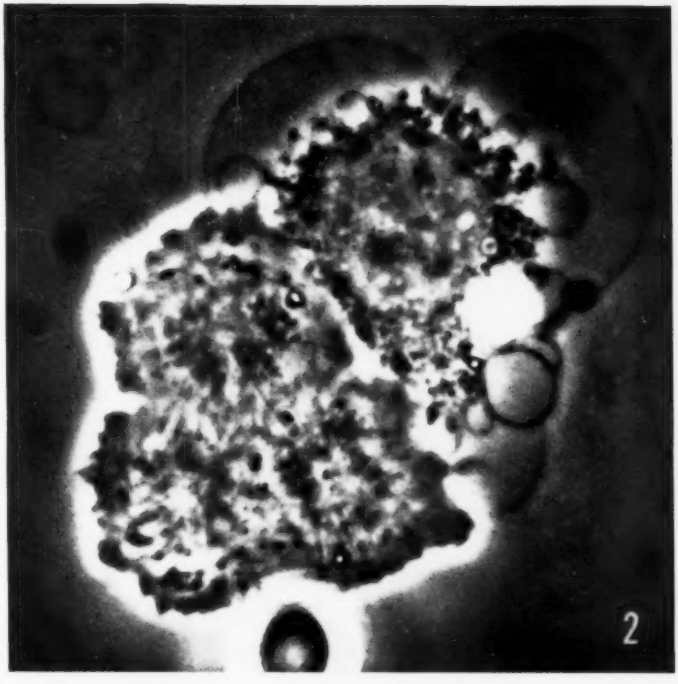


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PLATE 19

FIGURE 3.—A small sheet of mesothelial cells in their squamous form from a mouse 48 hours after the intraperitoneal inoculation of a suspension of melanin granules. The bright spot to the *left* is a droplet in the field.

FIGURE 4.—Neutrophilic granulocyte 18 hours after the intraperitoneal inoculation of a suspension of melanin granules. This cell is well flattened so the details are clearer than is usual. The cytoplasm contains several discrete melanin granules.

FIGURE 5.—An eosinophilic granulocyte from a control mouse. The nucleus is ring-shaped and the rodlike granules can be distinguished just inside the inner margin. The bright center is caused by strong refraction of light from the granules. This has obliterated the cytoplasmic detail.

FIGURE 6.—A mast cell from a control mouse. Some of the granules may be seen over the nucleus.

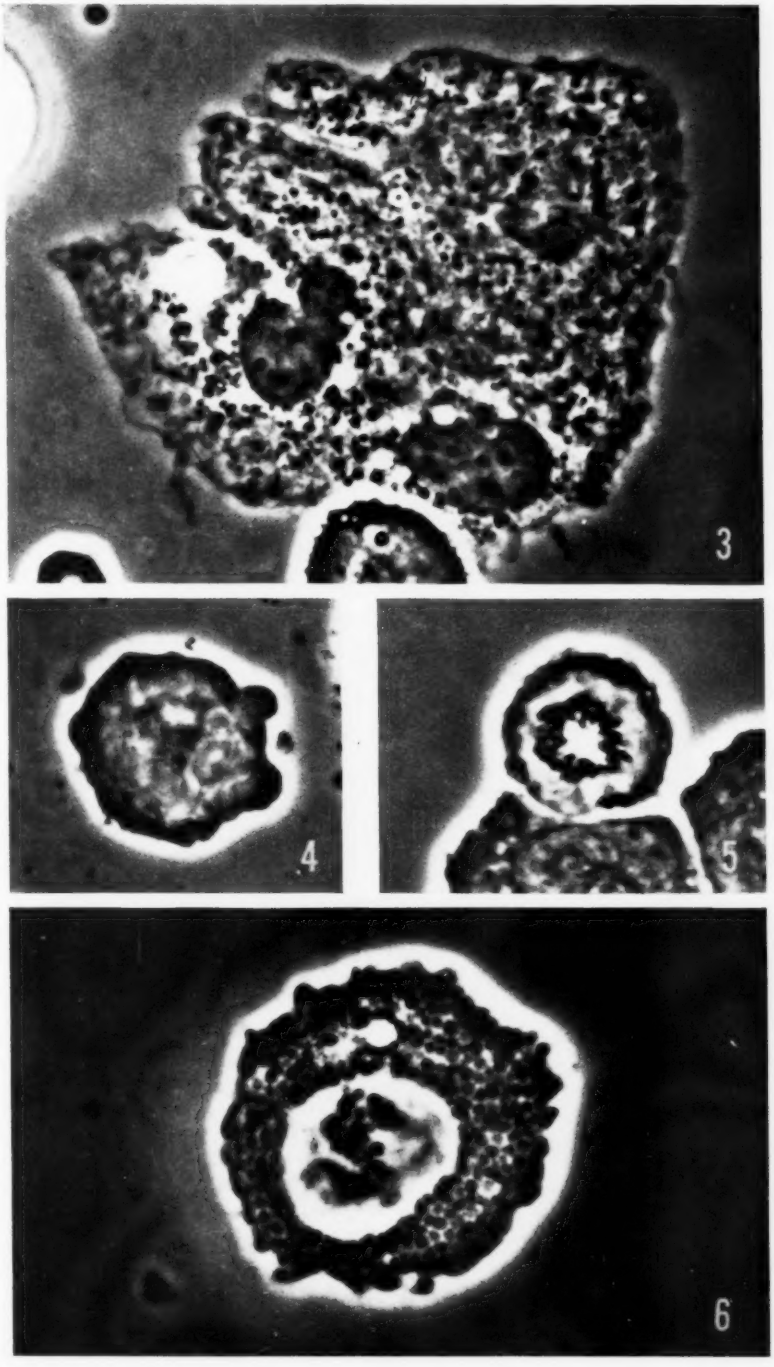
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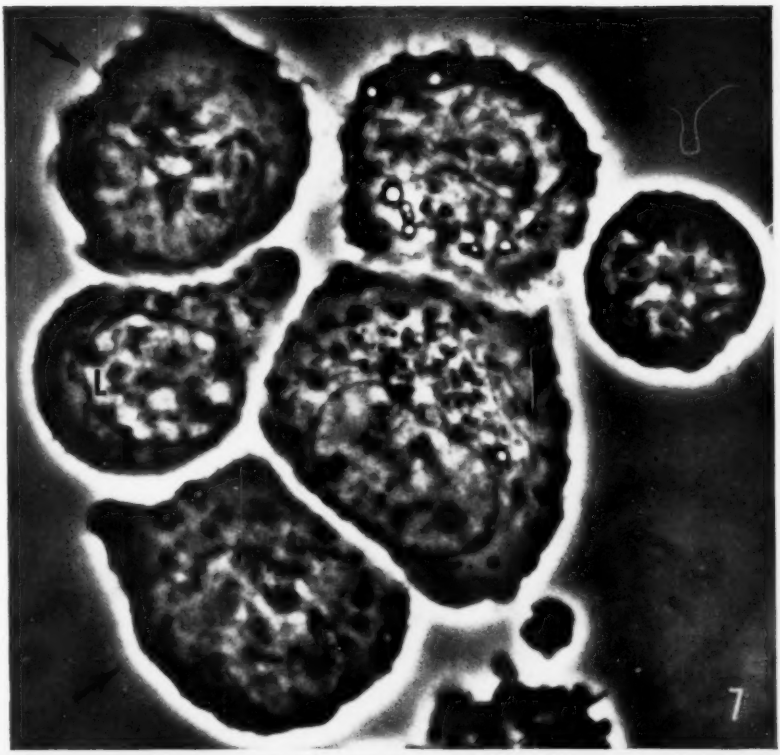
PLATE 20

FIGURE 7.—A group of cells 24 hours after the third intraperitoneal inoculation of 0.85 percent NaCl. In the field are two lymphocytes, and four cells in transition from lymphocyte to macrophage. Two cells (*arrows*) appear to be in the early stages of transition while the other two more nearly approach macrophages. Cytocenters are clearly distinguishable in the *upper left* cell and the cell in the *center*. A number of refractile droplets may be seen in the *top center* cell and several dark granules in the *center* cell.

FIGURE 8.—A macrophage with phagocytosed cellular debris and a mesothelial cell in squamous form with blebs at the cytoplasmic edges, 24 hours after the first intraperitoneal inoculation of 0.85 percent NaCl.

FIGURE 9.—Macrophage in metaphase from a mouse 21 days after the intraperitoneal inoculation of melanoma 891. Numerous spherical and filamentous mitochondria are visible.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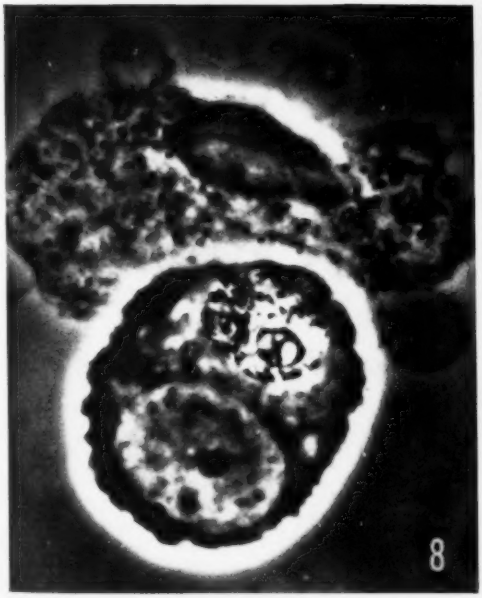
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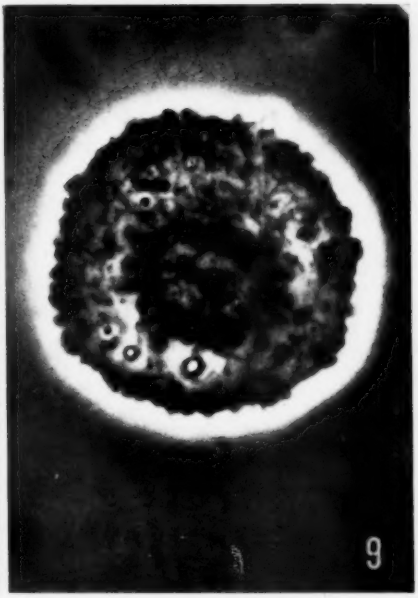
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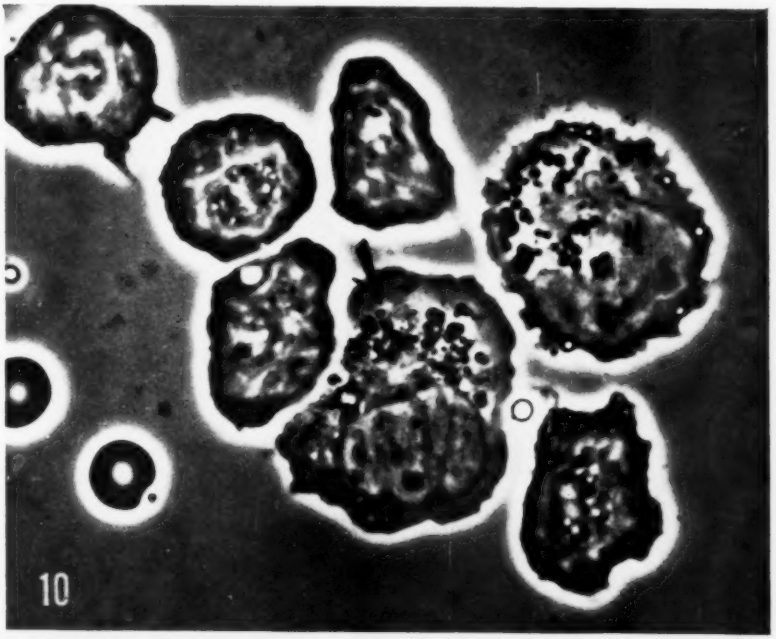
PLATE 21

FIGURE 10.—Two macrophages and five neutrophilic granulocytes from a mouse 24 hours after the intraperitoneal inoculation of a suspension of melanin granules. In one macrophage phagocytosed particles can be seen around the periphery of the cytocenter (*arrow*). The neutrophilic granulocytes contain one to several discrete melanin particles. The one at *top center* is blurred from movement. In the background of the field several free melanin particles may be seen. Two erythrocytes are visible at the *left* of the figure.

FIGURE 11.—A clump of three macrophages from a mouse 24 hours after the intraperitoneal inoculation of a suspension of melanin granules. The cytoplasm of two of them is well filled with vacuoles containing closely packed melanin granules. A small lymphocyte is in contact with the center macrophage at the *upper right*. At the *lower right* is a cell in early transition from lymphocyte to macrophage. It contains a few discrete melanin granules.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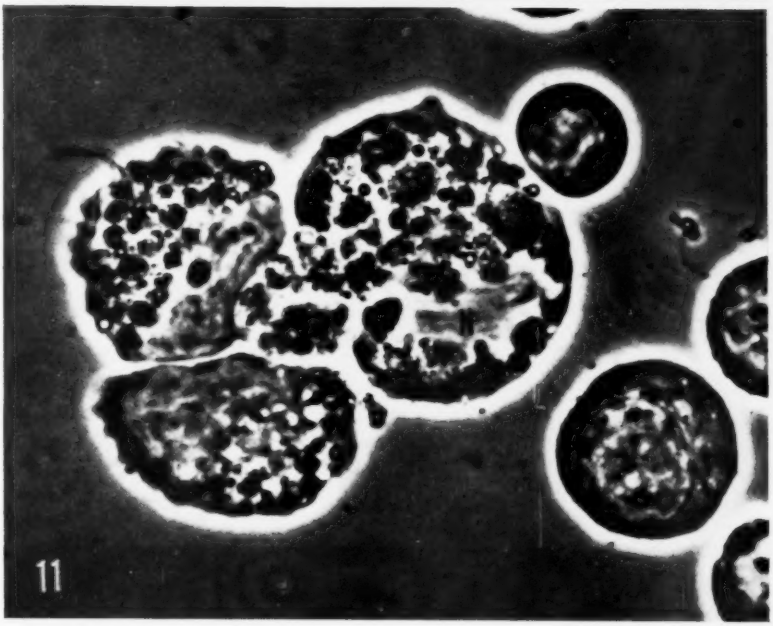
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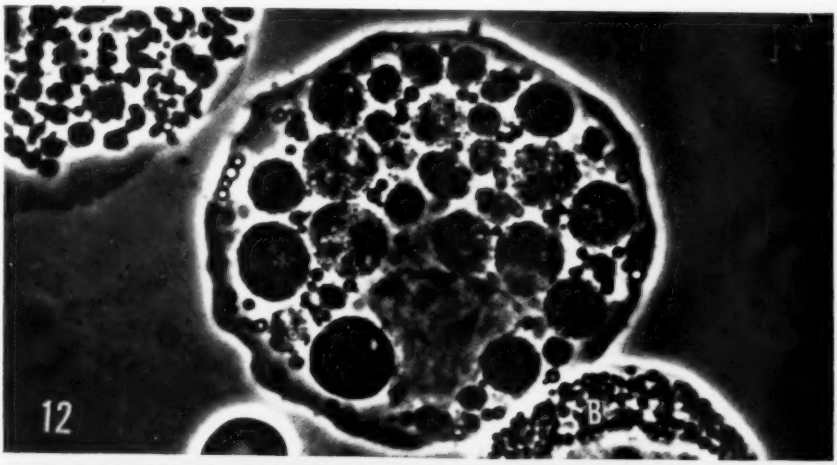
PLATE 22

FIGURE 12.—Macrophage from a mouse 48 hours after the intraperitoneal inoculation of a suspension of melanin granules. The cell is more flattened than those in the previous figure, showing the vacuoles containing closely packed melanin granules in greater detail. A few small refractile droplets may be seen. Part of a mast cell is also in the field at the *lower right* and, to the *lower left*, an erythrocyte.

FIGURE 13.—Very large macrophage from a mouse 4 days after the intraperitoneal inoculation of a suspension of melanin granules. Two vacuoles containing closely packed melanin granules and many vacuoles containing few melanin granules are present. The granules in the latter vacuoles are not clear because of Brownian movement. This cell is well flattened so that there is good nuclear detail but the cytoplasmic edges are distorted. Many mitochondria can be seen in the clear area near the nucleus. A number of refractile droplets are scattered throughout the cytoplasm. A smaller macrophage with vacuoles containing closely packed melanin granules is in contact with the larger cell at the *lower right* of the field.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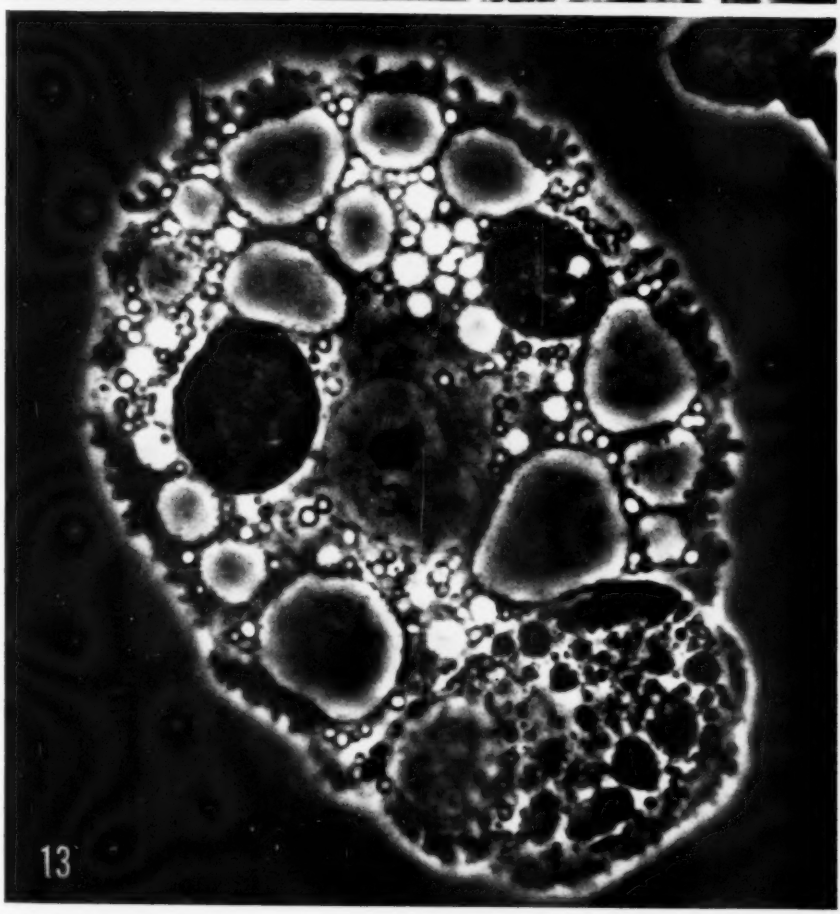
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PLATE 23

FIGURE 14.—Very large macrophage in a clump of macrophages from a mouse 7 days after the intraperitoneal inoculation of a suspension of melanin granules. Large vacuoles containing few melanin granules in Brownian movement are present as well as many large refractile droplets. This cell is well flattened but there is no marked distortion of the cytoplasmic edges.

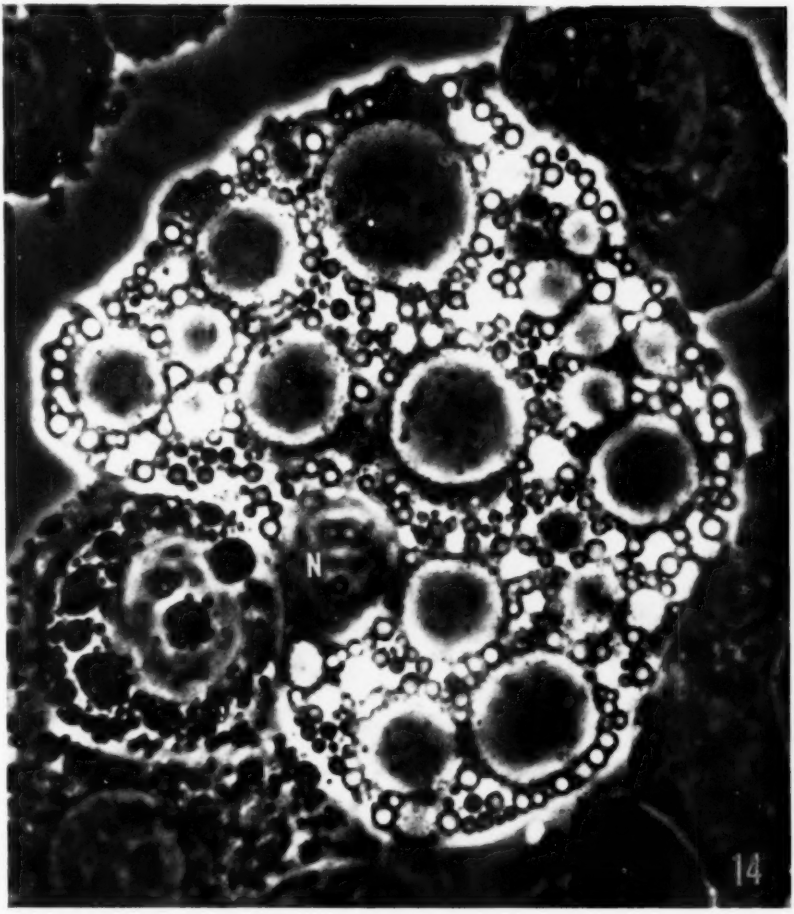
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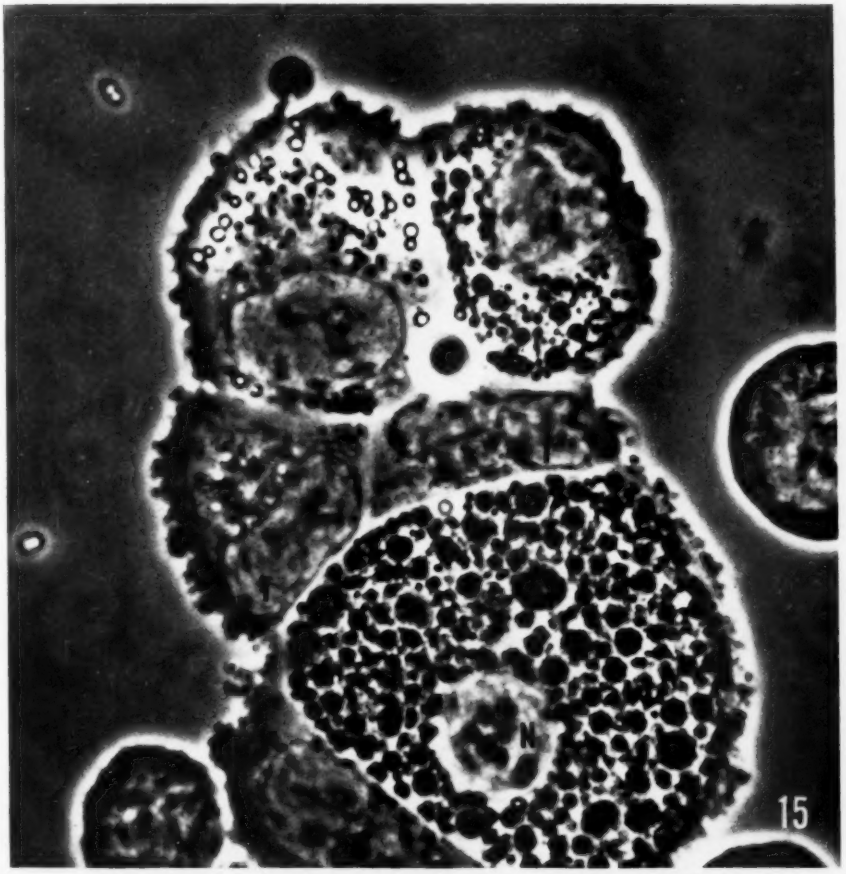
PLATE 24

FIGURE 15.—Group of macrophages 6 days after the intraperitoneal inoculation of a suspension of melanin granules. An illustration of the wide variation in size and form found during the response to inoculation with particulates, and of the tendency of the macrophages to form clumps. Two cells are in transition to macrophages and contain several discrete melanin granules. Mitochondria are particularly well defined in the transitional cell at *left center*. Parts of two lymphocytes are visible at the *lower left* and *right center*. A well flattened preparation with some distortion of the cytoplasmic edges.

FIGURE 16.—Macrophage in anaphase from a mouse 6 days after the intraperitoneal inoculation of a suspension of melanin granules. A number of vacuoles containing closely packed melanin granules are present.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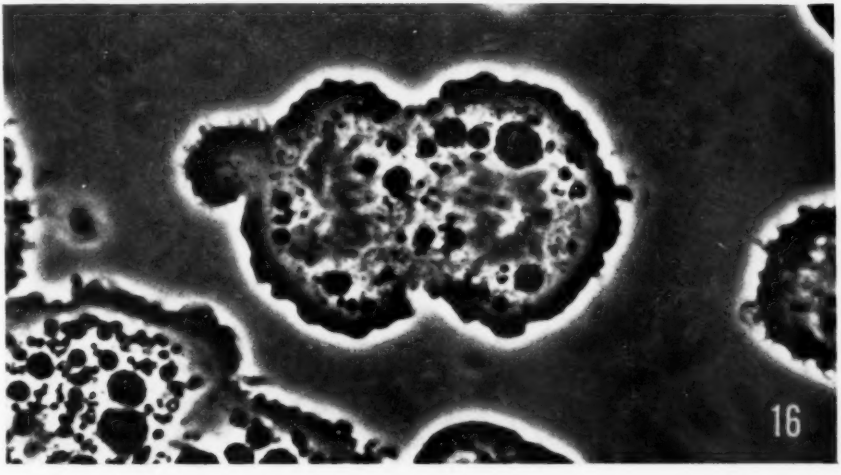
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PLATE 25

FIGURE 17.—Group of neutrophilic granulocytes 3 days after the intraperitoneal inoculation of a dilution of India ink. The cells contain carbon particles which are bright and irregular in shape, as well as refractile droplets and vacuoles. Two granulocytes have enspherulated and contain several large vacuoles. There are, at *top center*, one lymphocyte and several free carbon particles in the field, as well as an erythrocyte at *left center*.

FIGURE 18.—A thick preparation of a group of macrophages containing carbon particles from a mouse 24 hours after intraperitoneal inoculation of a dilution of India ink. In two of the cells a few of the particles are contained in large vacuoles. Two lymphocytes, at *upper left* and *lower right*, are in the field. At the *upper center* and *right edge* two neutrophilic granulocytes, blurred because of rapid movement, may be seen.

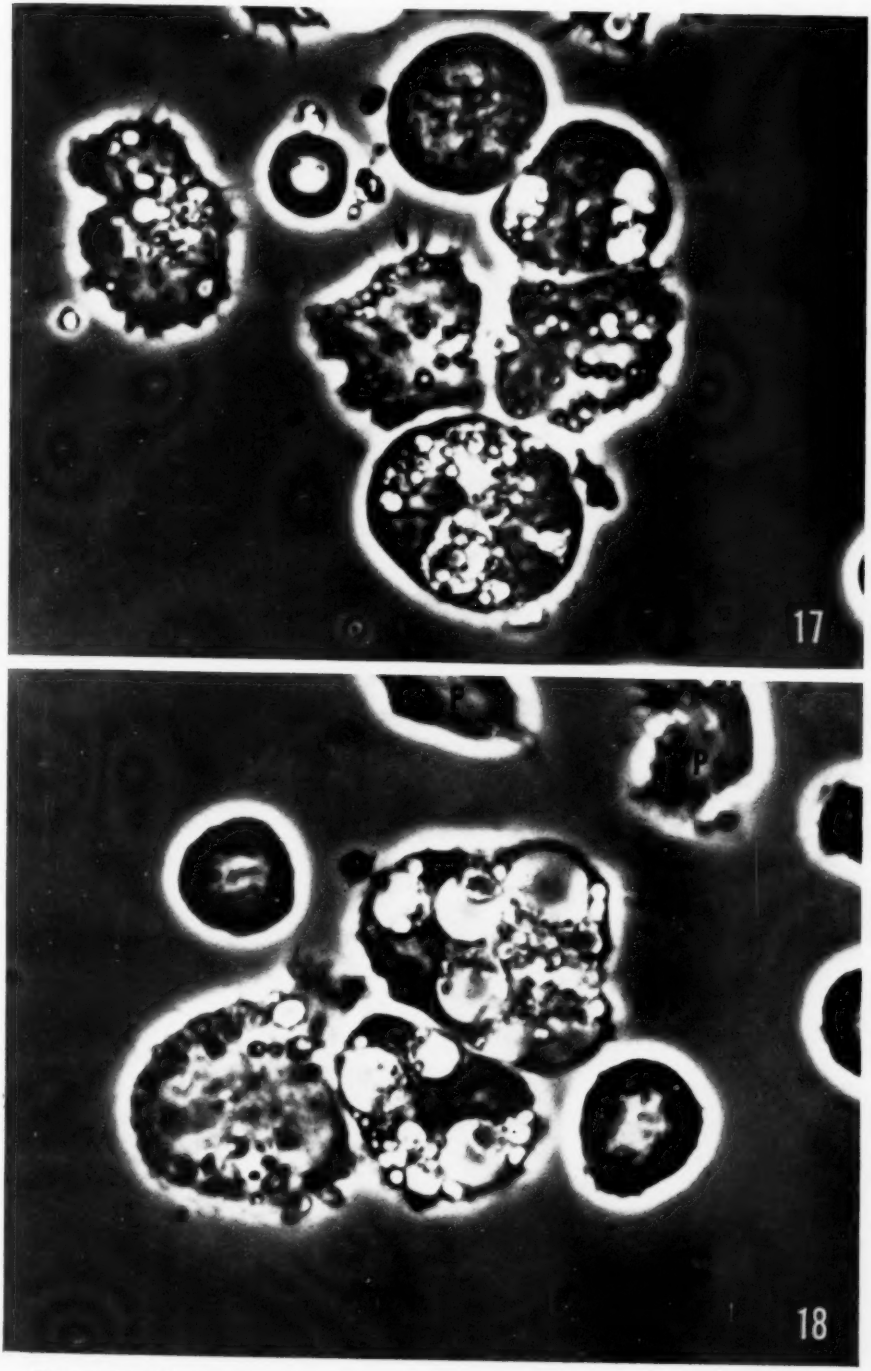
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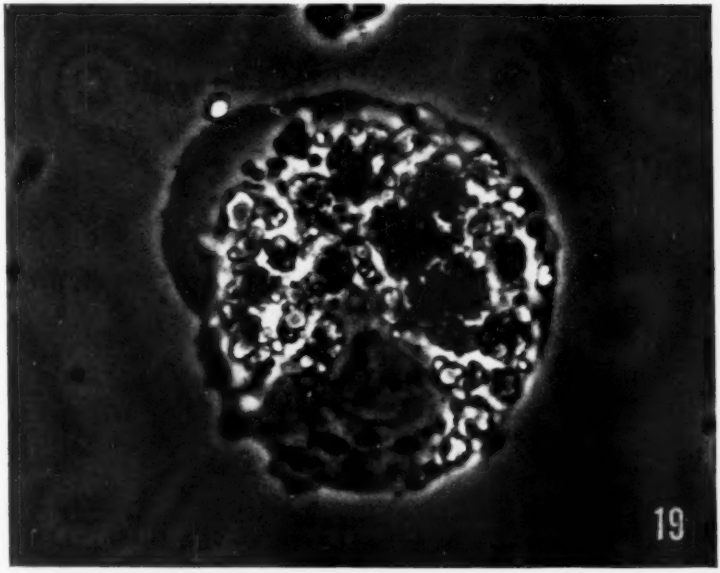
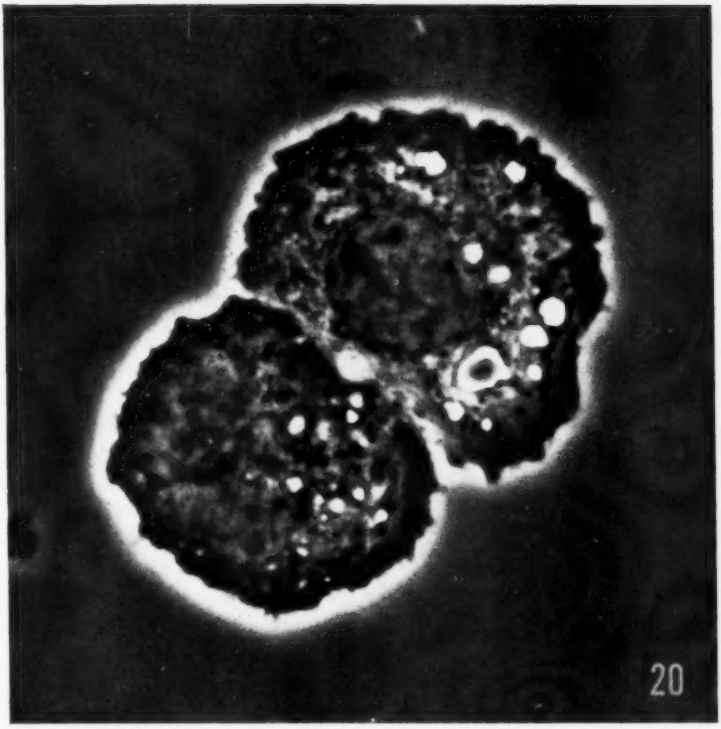
PLATE 26

FIGURE 19.—A large macrophage 4 days after the intraperitoneal inoculation of a dilution of India ink. The cell is well flattened to show the vacuoles containing closely packed, irregular carbon particles. Compare with the macrophage in figure 12. There is distortion of the cytoplasmic edges caused by pressure from the coverslip.

FIGURE 20.—Two macrophages 250 days after the intraperitoneal inoculation of a dilution of India ink. Both macrophages contain several ingested particles of carbon which appear bright.





352471—55——7

Experimental Studies in Metal Cancerigenesis. VII. Tissue Reactions to Parenterally Introduced Powdered Metallic Chromium and Chromite Ore[1]

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Observations made in German and American chromate plants have shown that chromate manufacturers have an excessive liability to cancer of the lung [Hueper (*1*), Mancuso and Hueper (*2*), Machle and Gregorius (*3*), Baetjer (*4, 5*), Gafafer *et al.* (*6*), and Gross and Koelsch (*7*)]. Similar hazards may exist for handlers of certain chrome pigments (zinc, barium, and lead chromates). However, no agreement has so far been reached as to the actual agent responsible for these occupational respiratory cancers. Not only has it remained controversial whether trivalent or hexavalent, soluble or insoluble chromium compounds, monochromates or bichromates, chromite ore or its intermediary roasting products are causally involved, but it is also still somewhat uncertain whether chromium and its compounds have any part in the carcinogenic process. Perhaps some unknown, associated factor present as an atmospheric pollutant from incomplete fuel combustion or representing a constituent of the ore, such as vanadium, iron, or silica, may play a significant primary or contributary role.

All experimental efforts made in the past to produce tumors in animals by the administration of chromium or its compounds by various routes (such as inhalation or intrafemoral implantation) either have failed or have given equivocal results [Gross and Alwens (*8*), Schinz and Uehlinger (*9*)].

The industrial production and use of chromium and its compounds has grown considerably during the past three decades. This development resulted in an exposure to these chemicals for large worker groups employed in the numerous industries and occupations manufacturing, processing, and using chromium, chromium alloys, and chromium compounds (stainless steel, chrome iron, leather tanning compounds, printing inks, paints, pigments for coloring glass, roofing shingles, linoleum and plastics, glazes of chinaware, catalysts used in the production of aniline dyes and in the catalytic cracking of petroleum oils, rocket fuel, refractory brick, anticorrosive coating of airplanes, antirusting agents in railroad

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

and automobile engines, industrial boilers and domestic steamheat plants). An attenuated form of contact with chromium and its compounds exists for those parts of the general population who use these chemicals in their homes or live near chromate-manufacturing plants or oil refineries discharging chromium-containing effluents into the environmental air and/or public waters.

Because of the potentially appreciable extent of an environmental chromium cancer hazard, as well as the prevailing uncertainty concerning the nature of the etiologic agent and its causal action mechanism, a comprehensive experimental investigation of metallic chromium and chromite ore, from a cancerigenic and immunochemical viewpoint, was included in a broad-based attack on metal cancerigenesis started several years ago [Hueper (*10*), Hueper, Zuefle, Link, and Johnson (*11*), Hueper (*12, 13*), Grogan and Oppenheimer (*14, 15*), Cahnmann and Bisen (*16*)]. Chromium and chromite ore were introduced in powdered form suspended in two different vehicles (lanolin, gelatin) by various routes (intrafemoral, intrapleural, intraperitoneal, intravenous, intramuscular, intranasal sinus) into mice, rats, guinea pigs, rabbits, and dogs. The tissue reactions to these procedures form the subject of the present report.

Experimental Procedures

The powdered chromium ore was obtained by the courtesy of Mr. O. F. Tarr, Vice President of the Mutual Chemical Company, Baltimore, Maryland. This material had been ground in a steel-ball mill to a fine powder of dark grayish-black color having a slightly greenish tint. It consisted of particles ranging in diameter from over 100 microns to colloidal-particle size. For intravenous injections, this powder was fractionated in a particle analyzer (American Instrument Company). The fraction composed of particles with a diameter of 4 microns or less was used for this purpose; for all other purposes the powdered chromite ore was employed in its original form. The pure powdered metallic chromium was purchased from the Chicago Apparatus Company. Since it also consisted of a mixture of particles having a wide range of diameter, a fraction of chromium particles measuring less than 4 microns in diameter was used for the purpose of intravenous introduction.

I. Chromium Experiments

1. Intrafemoral Implantation

A. Rats.—Two sets of experiments were done. In one experiment, 25 male rats of the Osborne-Mendel strain, about 5 months old, were injected into the marrow cavity of the right femur with approximately 0.2 cc. of a 50 percent by weight suspension of powdered chromium in lanolin (about 45 mg. of chromium). This operation was performed under ether anesthesia. The hole in the femoral cortex was drilled with an electric hobby drill having a flexible shaft and a #1 dental burr.

In the second experiment 25 male rats of the Wistar strain, about 5 months old, were used. The same operative technique was employed, and 0.2 cc. of an approximately 50 percent by weight suspension of pow-

TABLE 1.—*Survival periods of animals injected with chromium*

Site of injection	Species	Series	No.	Months								
				0–3	4–6	7–9	10–12	13–15	16–18	19–24	25–30	31–36
Intrafemoral....	Rat	I	25	2	8	1	—	1	4	9		
		II	25	—	3	—	3	3	6	10		
Intrapleural.....	Rat	I	25	2	3	—	6	3	5	6		
		II	25	—	—	1	2	—	3	7	12	
	Guinea pig	—	26	—	9	—	7	—	1	0	1	8
Intraperitoneal..	Rat	—	25	1	5	2	3	1	3	10		
Intravenous....	Rat	—	25	—	2	4	4	—	2	13		

dered chromium in a 20 percent gelatin-saline solution was injected into the right femoral cavity.

With the exception of an occasional abscess at the site of deposition of chromium, there were no acute surgical or toxicologic reactions. The last survivors of both series were sacrificed at the end of an observation period of 24 months (table 1).

The post-mortem examinations performed on these rats in most cases showed the presence of some soft, blackish material in the tissues surrounding the right femur. In only one of the Osborne-Mendel rats was a tumor found in the vicinity of the injected chromium. This rat had a firm, white, encapsulated mass with an indistinct, lobulated, cut surface involving the subcutaneous tissue of the right thigh and measuring about 4 cm. in diameter; upon histologic examination, it proved to be a cellular fibroma. With the exception of a minute adrenal cortical adenoma in a second Osborne-Mendel rat, no other neoplasms were found in the animals of this experiment.

The neoplastic yield obtained in the Wistar rat series was distinctly greater, although none of the various tumors found involved the region of the right thigh. One spindle-cell sarcoma and one cellular fibroma involved the region of the left groin and hip, respectively, while in a third rat a second spindle-cell sarcoma was found originating from a hepatic mass and extending into the mesentery, stomach wall, and heart (fig. 1). There were, moreover, two large round-cell sarcomas originating in ileocecal lymph nodes and associated with severe chronic inflammatory and ulcerative changes in the cecal wall (fig. 2). A causal relation between primary inflammatory changes in the cecum and the ileocecal lymph nodes was suggested by the fact that there were 8 other rats in this series which suffered from extensive chronic cecitis and markedly hyperplastic and, in part, cystic ileocecal lymph nodes. In some of these cystic nodes there was a complete loss of lymphoid structure which was replaced by diffuse masses of lymphoid and mononuclear cells. These cells were embedded in a highly edematous fibroblastic stroma surrounding large cystic spaces which had, in part, delicate fibrillar walls (fig. 3). Degenera-

tive changes of the liver cells and interstitial round-cell and fibroblastic infiltrations were found in many of these rats, one of which also had a large hemangioma of the adrenal medulla, while a second showed a pancreatic insuloma measuring 2 mm. in diameter (fig. 4).

B. Rabbits.—Eight rabbits of the small Dutch strain, 3 months of age and weighing from 1,350 to 2,100 gm., were injected through a drill hole into the right femur with about 0.4 cc. of a 35 percent by weight suspension of powdered chromium in lanolin. Each rabbit thus was given a single dose of about 30 mg. of chromium. After 30 months, four survivors were reinjected with twice the original dose of chromium delivered into the periosteal tissue of the right femur.

The rabbits died 3, 6, 19, 25, 33, 49, 50, and 58 months, respectively, after the first injection of chromium. At necropsy it was found that two rabbits had rather large, shallow ulcers of the gastric mucosa. One had a grayish-pink, polypous, soft tumor projecting from the diaphragmatic pleural surface into the right pleural cavity and measuring $2 \times 2 \times 1$ cm. The same rabbit showed a rather firm pinkish growth measuring about 3 cm. in diameter in the uterine cavity at the right junction of the uterine horn with the corpus. The vagina was filled with a soft, dark-red, polypous mass measuring 2 cm. in diameter. There was a swelling of the middle portion of the femur present in three rabbits, which, in one of them, was complicated by a large abscess encapsulated by a thick wall of baconlike tissue. In all rabbits blackish material was found embedded into the periosteal tissues of the right thigh.

The histologic examination of the tissues showed that the injected chromium apparently was retained in greatest quantity at the site of deposition, where it was found as a black powder embedded in clumps into fibrous tissue, or included, or finely distributed within mononuclear foam cells. Highly cellular, sarcomatoid granulation tissue was found in a cystically distended marrow cavity of the femur in only one rabbit. The various tumors noted in one rabbit were a papillary cystic adenoma of the endometrium, a hemangioma of the vagina, and a fibrolipoma of the pleura.

C. Dogs.—Five dogs of mixed breed, weighing from 6 to 8.5 kg., were injected into the marrow cavity of the right femur by the previously described method with 0.3 to 0.7 cc. of a 57 percent by weight suspension of chromium powder in lanolin. Twenty-one months later, twice this dose was injected into the periosteal tissue of the midportion of the right femur, where the first intrafemoral deposition of chromium had been made.

One of the dogs died 17 months after the first injection from the effects of an ether anesthesia. The post-mortem examination and histologic study of the organs of this dog revealed essentially normal conditions. The four surviving dogs are alive and well 60 months after the start of the experiment.

2. Intrapleural Implantation

A. Rats.—Two sets of experiments were done. In one set 25 rats of the Osborne-Mendel strain, 17 females and 8 males, approximately 4

months old, were injected through the right supraclavicular fossa into the pleural cavity with 0.05 cc. of a 33.6 percent by weight suspension of powdered chromium in lanolin. A total of 6 injections was made at intervals of 1 month.

In the second set, 25 Wistar rats, approximately 4 months old and of male sex, were intrapleurally injected through the right fifth intercostal space with 0.1 cc. of a 0.5 percent suspension of chromium in a 2.5 percent gelatin-saline solution. The powdered chromium consisted of particles measuring up to 4 microns in diameter. The injections were repeated 6 times at 1-week intervals.

The surgical procedures, described below, were well tolerated.

At autopsy, three of the female rats showed large, well encapsulated, nodular tumors, histologically of adenofibromatous structure, attached to the thoracic wall. One of these rats also had a large retroperitoneal mass with a multicystic cut surface. It consisted of a cellular fibroblastic network with endothelial cells lining the angiectatic cavities, which were filled in part by erythrocytes (hemangioma). Two other rats revealed large and firm multicystic tumors, one involving the right shoulder region, the other the left hip; both were firmly attached to the tissues at their base. Upon histologic examination, the one located at the shoulder proved to be angiosarcomatous in character and had formed multiple pulmonary metastases. The second was a fibrosing hemangioma. There was, in addition, one rat with a large round-cell sarcoma originating from the ileocecal lymph nodes and forming a fused, medullary, hemorrhagic mass. One rat had a large pituitary adenoma; another, a medullary angioma of the adrenal which measured 1 cm. in diameter (fig. 5). The lungs often showed chronic inflammatory changes with pseudoglandular cystic transformations of bronchioli. There was little tissue reaction to the injected metal within the thoracic wall and the axillary lymph nodes. The pleura was sometimes covered with soft, verrucous, black projections which consisted of vascular granulation tissue with numerous small cysts containing amorphous black matter.

Controls.—In a series of 12 male Wistar rats which received 6 weekly injections of the gelatin vehicle and which died or were sacrificed within 18 months from the start of the experiment, 3 showed at autopsy large, hemorrhagic, medullary tumor masses in the ileocecal region which consisted of large round-cell sarcoma tissue.

B. Guinea Pigs.—A series of 26 guinea pigs, 3 months old, were given 6 injections of 0.2 cc. of the 33.6 percent suspension of chromium in lanolin into the right pleural cavity at 1-month intervals.

Post-mortem examinations performed on these animals showed the presence of a hemorrhagic or seropurulent pleurisy in 12. The right lung often revealed a diffuse black mottling due to the presence of black, amorphous deposits in the subpleural tissue and the formation of soft, black, verrucous excrescences on the pleural surface. The histologic study did not yield any significant findings except for the occurrence of small and large black clumps in the interstitial tissue of the right lung in some

of the guinea pigs. This material was evidently a part of the injected chromium, which, by accident, was directly introduced into the lung, or which was secondarily absorbed into it by phagocytosis or lymph drainage from the pleural space. While in most cases there was little evidence of a local tissue reaction to these metal deposits, in some animals the clumps were embedded in a small amount of fibrous or hyaline tissue. In six guinea pigs which survived from 30 to 36 months, there was noted a marked proliferation of the bronchiolar epithelium. The bronchiolar lumina were then filled by a solid plug of hyperchromatic, oval-shaped epithelial cells which extended into the lumina of the adjacent alveoli.

C. Mice.—Fifty strain C57BL male mice, approximately 6 weeks old, were injected into the right pleural cavity with 0.2 cc. of a 0.005 percent chromium suspension in a 2.5 percent gelatin-saline solution. The injections were repeated 6 times at 2-week intervals. Thirty-two mice survived from 7 to 14 months. Except for pneumonic and pleuritic reactions in many of the mice, there were no significant pathologic lesions. Tumors were not observed.

3. Intramuscular Implantation

Mice.—Twenty-five strain C57BL male mice, approximately 6 weeks old, were given 2 injections, 2 weeks apart, of 0.02 cc. of a 0.005 percent suspension of chromium in a 2.5 percent gelatin-saline solution into the muscle tissue of the right thigh. This procedure was repeated 3 weeks later. Fifteen mice survived from (at least) 6 months up to 13 months.

Tumors did not develop in any of the mice. The intramuscular chromium deposits were usually without any appreciable cellular or fibrosing reactions.

4. Intraperitoneal Implantation

A. Mice.—Fifty strain C57BL male mice, approximately 6 weeks old, were intraperitoneally injected once a week for 4 consecutive weeks with 0.2 cc. of a 0.005 percent suspension of chromium in a 2.5 percent gelatin-saline solution.

Forty mice survived from 6 to 21 months. With the exception of a myeloid leukemia found in a mouse which died 1 year after the first injection no other neoplastic reactions were noted.

B. Rats.—Twenty-five male Wistar rats, 3 to 4 months old, were intraperitoneally injected once every week for 6 consecutive weeks with 0.1 cc. of a 0.05 percent chromium suspension in a 2.5 percent gelatin-saline solution.

At autopsy, two rats showed enlarged and fused ileocecal lymph nodes which were hemorrhagic and medullary and measured up to 4 cm. in diameter. Similar nodes were found in the mesentery, the retroperitoneal space, at the hilum of the liver, in the spleen, and also attached to the gastric serosa, the wall of the colon, the pancreas, and the mediastinum. Histologically they were composed of solid masses of large round cells em-

bedded in a loose, edematous, vascular, and hemorrhagic stroma (round-cel-sarcoma). A third rat had a markedly thickened and elongated appendixl like projection of the cecum. It measured 3 cm. in length and had a reddish-white, verrucous wall measuring 1 cm. in thickness. There was, histologically, a large mucosal ulcer, the floor and margins of which consisted of irregular glandular formations. The submucosa was markedly thickened and fibrotic. The fibrotic tissue extended into the muscularis and was diffusely infiltrated with small strands and groups of hyperchromatic epithelial cells characteristic of a scirrhus carcinoma (fig. 6). In a fourth rat, the right foreleg was swollen and covered with a crust, underneath which was found a white medullary tissue attached to the bone. The tumor measured 2.5 cm. in diameter and consisted histologically of large, round cells apparently originating from a proliferating, partly cartilagenous, partly osteoid trabecular periosteal tissue in the meshes of which groups of hyperchromatic sarcoma cells were found. The round-cell sarcoma invaded the adjacent muscle tissue (fig. 7). In addition, the same rat had a light-stained, well-encapsulated node in the pancreas measuring 1.5 mm. in diameter and exhibiting the characteristic structure of an insuloma (fig. 8). A second insuloma was discovered in a fifth rat. This measured 1.5 mm. in diameter and was composed of light-colored cells arranged partly in garlands, partly in a glomerular pattern (fig. 9).

5. Deposition of Chromium in the Paranasal Sinuses

Twenty female Wistar rats, about 3 months old, were injected with a 33.6 percent by weight chromium suspension in lanolin into the right nasal sinuses by perforating the lamina cribiformis with a needle introduced into the right nasal cavity. Each dose (0.1 cc.) was given twice, with an interval of 1 month between the injections.

Two rats died during the first 6-month period, 2 during the second, 8 during the third, and 8 during the fourth. One rat had a well-encapsulated, lobulated, white tumor in the right groin which was histologically an adenofibroma. This rat and 5 others had medullary hemangiomas of the adrenal glands. A seventh rat showed a large, nodular, fused, hemorrhagic, and medullary whitish mass in the ileocecal region, which histologically exhibited the structural characteristics of a large round-cell sarcoma. Metastatic nodes were found in the pancreas, spleen, mesenteric lymph nodes, and subserosa of the stomach. An eighth rat displayed a deep-red and enlarged hypophysis which was histologically a basophilic adenoma. The same rat also had ill-defined foci of giant liver cells, apparently representing regenerative adenomatoid proliferations, since distortion of the normal liver cell arrangement and a diffuse interstitial infiltration with lymphocytes were found. The mucosa and submucosa of the paranasal sinuses contained black, amorphous deposits and a leukocytic infiltration. In several rats there was also a proliferation of the lining epithelium, associated with irregularities in shape, size, and arrangement of the cells, as well as an appreciable increase in oval- and spindle-shaped fibroblastic cells of the submucosa. The lumina of the

sinuses usually were filled with a leukocytic exudate. No neoplastic reactions were noted.

6. Intravenous Injection of Chromium

A. Mice.—Twenty-five C57BL mice, about 8 weeks old, were injected into the tail vein with 0.05 cc. of a 0.005 percent suspension of chromium in a 2.5 percent gelatin-saline solution once a week for 6 consecutive weeks. Six of the mice died during the first 6-month period, 13 during the second, and 6 during the third. None of the mice showed any tumor formation at autopsy or upon histologic examination of the organs. In mice which died within a few hours after an injection of chromium, brownish-black, amorphous matter was found within the capillary lumina and the perivascular tissue of the lung and kidney.

B. Rats.—Twenty-five male Wistar rats, approximately 7 months old, were injected into the left vena saphena with an average of 0.18 cc. of a 0.05 percent suspension of powdered chromium in a 2.5 percent gelatin-saline solution once a week for 6 consecutive weeks. Blood examinations (red-cell, white-cell, and differential counts, hemoglobin) performed before and 2 months after the first injection revealed no significant effects upon these elements from the injected material.

Three rats exhibited, at autopsy, medullary and hemorrhagic nodular masses in the ileocecal region, with secondary extensions into the mesenteric, retroperitoneal, mediastinal, and axillary lymph nodes, subcutaneous tissue, spleen, liver, and pancreas. The ileocecal masses measured up to 10 cm. in diameter. All of them consisted of large round-cell sarcomas. In a fourth rat a histologically identical sarcoma involved the mediastinal lymph nodes, lung, and heart. A fifth rat had a hemangioma of the adrenal medulla measuring 3 cm. in diameter. The angiectatic cavities were filled with masses of hyperchromatic oval cells (fig. 10). In two other rats, upon histologic examination, ill-defined papillary adenomas involving the alveolar structures of the lungs were noted. One measured 1 cm. in diameter (fig. 11), while the second was 2.5 cm. in diameter. In this tumor, there was an extensive metaplastic transformation of the cylindrical epithelium into a squamous-cell carcinoma which in places infiltrated in solid nests and strands the surrounding pulmonary tissue and which revealed the histologic characteristics of a squamous-cell carcinoma (fig. 12). In some of the lungs there were brownish-black, amorphous deposits in the lumina of pulmonary capillaries and in the interstitial pulmonary tissue. This condition was associated in one rat with multiple small, calcified foci in the pulmonary tissue.

C. Rabbits.—Eight albino rabbits, about 6 months old, were injected into the ear vein with 0.5 cc. per kg. of body weight of a 5 percent suspension of powdered chromium in 2.5 percent gelatin-saline solution which was given once a week for 6 consecutive weeks. This course of treatment was repeated 4 months later. Three years after the first injection a third series of injections was administered to 3 surviving rabbits. Some of the injected chromium settled in the venous lumina and formed black

indurations obliterating the lumina. The injections were otherwise well tolerated.

Four rabbits intravenously injected with the gelatin-saline vehicle in the same fashion as the 8 test rabbits served as controls.

Of the 5 rabbits which have come to autopsy, none have shown any neoplastic lesions. The 3 surviving rabbits appear to be normal. Blood studies (leukocyte, erythrocyte, and differential counts, hemoglobin, coagulation, and bleeding times) performed at various intervals showed an increase of the bleeding time (Duke's method) from a pre-experimental average value of 1 minute 58 seconds to about 3 minutes after 3 years. However, a similar fluctuation was observed in the control rabbits given gelatin solution only.[3]

However, with respect to some tumors noted in rats given metallic chromium powder by various routes, one must consider the possibility of a causal connection between a biologic action of this metal and the development of neoplasms, since in previously reported experiments similar tumors were absent among control animals as well as among rats given other metals and minerals by the same routes. Among the cancers of suspected or possible chromium etiology may be included the three spindle-cell sarcomas present in Wistar strain rats intrafemorally injected with this metal and the three hemangiomatous tumors found among the rats receiving intrapleural injections of this material, as well as the round-cell sarcoma of the leg and the adenocarcinoma of the intestine noted in rats given intraperitoneal administrations of chromium. There is, moreover, a distinct possibility that the papillomatous tumors of the lung observed in two rats after an intravenous introduction of chromium may have had a metal-specific genesis. A similar suspicion may be maintained in regard to a chromium-specific origin of the three insulomas, two of which developed in rats given chromium by the intraperitoneal route, while a third was present in a rat receiving this metal by intrafemoral injection.

Although the present experimental investigations have not yielded any evidence resembling that provided by Schinz and Uehlinger (*9*) on rabbits, they offer at least a few observations suggesting that parenterally introduced chromium may have played a part in the development of a few tumors near as well as remote from the site of introduction.

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[3] After surviving 3½ years of experimentation, one of the 3 rabbits given a third series of intravenous chromium injections died 6 months following this procedure. Firm, white, medullary, nodular masses measuring up to 5 cm. in diameter were present in the axillary and inguinal regions of both sides, along the mammary line on the right side, in the lungs, submaxillary gland, mediastinal and retroperitoneal lymph nodes, liver, ovaries, kidneys, adrenal glands, and walls of the stomach and intestine. The histologic examination of the various tissues showed large clumps of amorphous, black material in the hepatic capillaries and splenic sinuses as well as in the stroma of a tumor mass involving a lymph node. The tumor tissue consisted of solid masses of rather small round cells with ill-defined cellular outlines, exhibiting in places a tendency toward an abortive glandular structure and rosette formations. Penetration of the tumor tissue into the lumina of large veins was observed in several places. Cancerous infiltrations were found in various lymph nodes, in tissues of the mammary region, submaxillary glands, lungs, liver, kidney, omentum, adrenal glands, ovaries and meningeal membranes. The tumor is apparently an immature carcinoma, probably of glandular origin. Its generalized distribution and its anaplastic structure, however, interfere with the positive determination of its tissue of origin, which most likely is either the ovaries, the mammary glands, or the submaxillary gland. Its relation to the injected chromium remains uncertain for the same reasons.

II. Chromite Experiments

1. Intrafemoral Implantation

A. Rats.—The chromite ore employed was finely powdered Transvaal ore which contained about 44 percent of Cr_2O_3 by assay and which also contained iron, vanadium, silicon oxide, aluminum oxide, and magnesium oxide; this material was suspended in lanolin at a ratio of 50 percent by volume or 73.4 percent by weight. Twenty-five 5-month-old Osborne-Mendel rats (15 males, 10 females) were injected into the marrow cavity of the right femur with 0.05 cc. of the suspension containing about 58 mg. of chromium ore. The injections were well tolerated. Over a 24-month observation period, 3 rats died during the first 6 months, 7 during the second, 8 during the third, and 5 during the fourth.

One of the rats showed at autopsy a diffuse thickening of the midportion of the femur. In two other rats there existed in the right thigh shallow ulcerative lesions leading into fistulous ducts which connected with swollen and soft osteomyelitic femoral bones. The tissue surrounding the ducts was white, medullary, and firm. Blackish deposits were present in the periosteal and surrounding muscular tissue of the right thigh. In one of the three rats with osteomyelitic lesions there was a yellowish-white hemorrhagic mass in the mesentery extending to the hilum of the liver.

Upon histologic examination of the tissues, the abdominal tumor proved to be a large round-cell sarcoma. The lesions of the femurs and the medullary tissue found around the fistulas were of chronic inflammatory nature. The chromite ore represented by the black deposits usually was embedded in a hyaline matrix. There were no other pertinent pathologic alterations in any of the rats.

B. Rabbits.—About 0.2 cc. of the chromite-ore suspension in lanolin was injected through a drill hole into the marrow cavity of the right femur of four Dutch rabbits. One died 20 months after the injection, a second 48 months later; and the last 2 were sacrificed 50 months from the start of the experiment. One of the last 2 rabbits showed at autopsy a marked swelling of the right uterine horn which measured $3 \times 2 \times 2$ cm. Upon dissection, a soft, pedunculated tumor with a smooth, yellowish-white surface was found in the uterine lumen.

The histologic examination revealed the presence of large masses of black, amorphous material embedded in the fibrotic and hyaline-thickened periosteal tissue. The uterine tumor noted in one rabbit was an endometrial adenocarcinoma.

2. Intrapleural Deposition

Twenty-five Osborne-Mendel rats, about 4 months old (14 males, 11 females), were injected once a month during 6 consecutive months into the right pleural cavity through the supraclavicular fossa with approximately 0.05 cc. of a 73.4 percent by weight suspension of chromite ore in lanolin. Three rats died during the first 6-month observation period, 9

during the second, 6 during the third, and 7 during the fourth. Blood studies performed at varying intervals did not reveal any abnormal reactions.

At autopsy, two female rats showed subcutaneous, well-encapsulated, nodular, firm tumors measuring 5 to 6 cm. in diameter in the right axillary region. In one of the male rats, white, medullary nodules, from pinhead to rice-seed size, were found attached to the inner surface of the xyphoid process, while flat white plaques were found covering the pleural aspect of the diaphragm. Larger medullary nodes involved the pectoral muscle of the right side, spleen, liver, subserosa of the stomach, and mesentery. Soft reddish tissue extended along the spermatic cord from the abdominal cavity down into the scrotum of a fourth rat which also had white peribronchial markings. Soft, black, amorphous matter was found in the right pleural cavity, axillary region, and thoracic wall of most rats.

Histologic studies showed the two subcutaneous tumors of female rats to be typical adenofibromas of the breast. The thoracic and abdominal tumors in one of the male rats consisted of fibrosarcoma tissue. The reddish perispermatic tissue in a second male rat was composed of papillary or polypous structures having mucinous central cores lined by a single layer or multiple layers of small cuboidal or oval cells (fig. 13), such as those seen in malignant synoviomas. The lungs, on the other hand, showed diffusely infiltrating peribronchial, irregular glandular formations surrounding distended and inflamed bronchi (fig. 14). The black deposits in the axillary region and covering the right pleura showed large clumps of black, amorphous matter and black, extracellular and intracellular powdery material, sometimes embedded in a loose vascular tissue, sometimes surrounded by a fibrous and hyaline tissue, and containing scanty nuclei.

3. Deposition of Chromite Dust into the Paranasal Sinuses

Twenty female Wistar rats, about 2 months old, were injected through the nares into the right paranasal sinuses with 0.1 cc. of a lanolin-chromite ore-dust suspension containing 49 percent by weight of chromite ore. One rat died during the first 6-month period, a second during the second, 9 during the third, and 9 during the fourth.

Two rats had subcutaneous nodular adenofibromas of the breast. In one of them there was also a medullary hemangioma of one adrenal gland. A similar adrenal tumor existed in a third rat. The liver of a fourth rat was nodular and showed, upon histologic examination, an increase in interstitial connective tissue which was infiltrated with mononuclear cells and proliferated bile ducts. In one area a small hemangioma was found. A fifth rat exhibited large, medullary, white, and hemorrhagic fused masses of lymph nodes in the mediastinum, mesentery, and ileocecal region, which were composed of large round-cell sarcoma.

The examination of the paranasal sinuses revealed the presence of brownish-black, amorphous matter in the submucosa and periosteal tissue, associated with inflammatory and fibroblastic reactions.

4. Intravenous Administration of Chromite-Ore Dust

Six female albino rabbits, about 6 months old and weighing between 3,250 and 3,750 gm., were injected once a week for 6 consecutive weeks into the ear vein with 5 cc. of a 5 percent suspension of chromite-ore powder in a 2.5 percent gelatin-saline solution. This course of injections was repeated 9 months later (on 4 rabbits). The 6 rabbits died or were sacrificed 13, 20, 22, 22, 48, and 48 months, respectively, after the start of the experiment.

Blood examination of these injections made at short and long intervals revealed that the intravenous injection of the chromite-gelatin suspension elicited a marked and acute colloidoclastic crisis of the blood, as evidenced by the appearance of a marked leukopenia with reduction of polymorphonuclear cells in the peripheral blood within 5 minutes after the injection, followed by a reactive leukocytosis approximately 30 minutes afterward.

The post-mortem examinations performed on these rabbits showed that in 3 there was hyperplasia of the endometrium, associated with a cystic distension of the uterine horns, which were filled with turbid, mucinous material. None of the animals showed any tumors. Histologic examination of the organs revealed the presence of large and numerous intravascular casts of brownish-black, amorphous, powdery, and clumpy matter within the capillary lumina of the liver and within the capillaries and sinusoids of the spleen. None were seen in the pulmonary vessels. Occasionally, there were a few casts of this type in the capillaries of the interstitial renal tissue. Powdery, blackish matter was found in some of the reticuloendothelial cells of the liver and spleen. The absence of any reactive changes around these deposits was remarkable.

III. Vehicle Control Experiments

In addition to the control experiments previously recorded, the following vehicle- and strain-control series are available for comparison with the test series (table 2).

The doses of vehicle (lanolin, gelatin, saline) administered, as well as the method of introduction, were identical with those used in the corresponding test series.

Of the total number of 165 control rats, 140 belonged to the Wistar strain, while 25 were of the Osborne-Mendel strain. Ninety-eight of them were females and 67, males. A total of 49 tumors of malignant or benign character were found among them (23 cancers). The most frequent tumors were round-cell sarcomas of the ileocecal lymph nodes, present in 14 rats. Only 1 cancer, a spindle-cell sarcoma involving the thigh, was found at the site of deposition of the vehicle (lanolin). The various tumors originated from 10 different tissues and/or organs. It is most unlikely that, with the exception of the spindle-cell sarcoma of the thigh, there was any causal relationship between the vehicle introduced and the tumor formed.

TABLE 2.—*Control series of rats given lanolin, saline, or gelatin*

Site of depot	Vehicle	Strain	Sex	Number of animals	Site of tumor	Number of tumors	Type of tumor
Intrafemoral	Gelatin	Wistar	15 males 15 females	30	Breast Ileocecal lymph nodes Liver	1 1 2	Adenofibroma Round-cell sarcoma Spindle-cell sarcoma
Intrafemoral	Lanolin	Wistar	23 females	23	Breast Adrenal Hypophysis Ileocecal lymph nodes Liver-spleen	4 2 3 2 1	Adenofibroma Hemangioma of medulla Basophilic adenoma Round-cell sarcoma Leukemia
Intrafemoral	Lanolin	Wistar	25 males	25	Thigh Mediastinal lymph nodes Liver	1 2 1	Spindle-cell sarcoma Round-cell sarcoma Hemangiosarcoma
Intrapleural	Lanolin	Osborne-Mendel	25 females	25	Breast Ovary Mesenteric lymph nodes Stomach	4 2 1 1	Adenofibroma Cystadenoma Spindle-cell sarcoma Squamous-cell papilloma
Intrapleural	Saline	Wistar	12 males	12	Ileocecal lymph nodes	4	Round-cell sarcoma
Intranasal sinus	Gelatin	Wistar	15 males 15 females	30	Ileocecal lymph nodes Liver Hypophysis	4 2 1	Round-cell sarcoma Round-cell sarcoma Basophilic adenoma
Intranasal sinus	Lanolin	Wistar	20 females	20	Adrenal Breast Hypophysis	5 2 2	Hemangioma of medulla Adenofibroma Basophilic adenoma

General Comments

The rationale underlying the experimental scheme described was twofold: 1) to find a species and tissue susceptible to the carcinogenic action of chromium and/or its compounds; 2) to ascertain whether or not the carcinogenic effect apparently associated with an occupational exposure to chromium compounds is exerted by chromium metal or some compound of low solubility, such as chromite ore or one of its intermediary oxidation products formed during the roasting process [Mancuso and Hueper (*2*), Gafafer *et al.* (*6*)]. Since histochemical and chemical investigations on the lungs of chromate manufacturers had shown the presence of considerable amounts of chromite-ore dust [Mancuso and Hueper (*2*), Hueper (*17*)] forming an intrapulmonary depot from which chromium might be released over long periods of time, it was reasoned that similar exposure conditions might be produced in animals by a parenteral introduction of such materials.

The results obtained are definitely equivocal, if not negative, as to evidence supporting a carcinogenic action of metallic chromium and chromite ore. The tumors for which the existence of such a relationship might possibly be entertained are: the 3 spindle-cell sarcomas and 1 insuloma noted in rats with intrafemoral chromium deposits; the 3 hemangiomatous tumors found at various sites in rats given chromium metal into the pleural cavity; the adenocarcinoma of the colon; the osteogenic round-cell sarcoma of the foreleg; the 2 insulomas of the pancreas observed in rats following a repeated intraperitoneal administration of chromium; 2 papillary adenomatous tumors, one of which showed extensive squamous-cell carcinomatous changes, in rats given chromium into the veins; a fibrosarcoma of the thoracic wall; and an adenocarcinoma of the lung, associated with a malignant synovioma of the spermatic cord, in 2 male rats given chromite ore intrapleurally.

Favoring such a concept is the fact that several tumors developed at or near the site of deposition of chromium and that several other neoplasms found distant from these depots were of distinctly unusual variety, *i.e*, two papillary adenomas of the lung [Horn and Stewart (*18*)] and three insulomas [Hueper (*13*)]. It may be argued, moreover, that the low tumor yield and the apparent shift in target organ may possibly reflect a distinctly lower susceptibility of rats to the carcinogenic action of chromium than that possessed by human beings. Experimental studies with aromatic amines, especially β-naphthylamine, benzidine, and 4-aminodiphenyl, have provided good illustrations attesting to the fact that tumor yield, length of latent period, and target organ may differ widely among different species for the same carcinogen [Hueper (*19*)].

In this connection, perhaps mention should be made of the fact that the yield of chromium-suspect tumors was definitely higher among the various rat series implanted with metallic chromium powder than among those with chromite-ore deposits. If these differences should prove to be attributable to variations in the relative amounts of available chromium in the two groups, the experimental conditions present support such a

concept. The animals in the chromite-ore experiments received approximately half as much chromium as those given metallic chromium; furthermore, only a small fraction of chromium in the chromite ore was soluble and, therefore, capable of biologic activity.

There are, on the other hand, several weighty reasons which militate against the assumption of a causal relationship between the chemicals administered and the development of the particular tumors listed. The total yield of tumors of this connotation is small, thereby allowing for the strong possibility of their coincidental occurrence. It is noteworthy, moreover, that all these tumors were found in rats only, none in rabbits, guinea pigs, or mice. In fact, none of the rabbits developed tumors similar in type and location to those previously reported by Schinz and Uehlinger (*9*) following an intrafemoral implantation of powdered metallic chromium, although some of the animals which died had survived for 4 years, while others are still living and apparently healthy, having completed their fifth year of observation.

Summary

1) Single parenteral administrations or repeated depositions of powdered metallic chromium and chromite ore into the femoral bone marrow, peritoneal cavity, pleural space, paranasal sinuses, skeletal muscle, and vascular lumen of mice, rats, guinea pigs, rabbits, and dogs were followed by the development of a relatively small number of tumors with uncertain causal relation to the metallic material implanted. A few of these neoplasms appeared at or near the site of deposition, while others, found remote from such locations, were of unusual histogenetic types, suggesting a possible causal connection. All neoplasms of these designations occurred in rats, none among the other species used.

2) In view of the foregoing observations, the appearance of 3 insulomas in rats given chromium intrafemorally or intraperitoneally and of 2 papillary adenomas of the lungs, 1 with squamous-cell carcinomatous changes, in rats given chromium intravenously was of particular interest.

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Plate 27

Figure 1.—Oval- and spindle-shaped sarcoma cells invading the myocardium of a rat. × 355

Figure 2.—Large round-cell sarcomas infiltrating lymphocytic tissue of an ileocecal lymph node of a rat. × 355

Figure 3.—Chronic cystic lymphadenitis with scattered large mononuclear cells involving ileocecal lymph nodes of a rat. × 355

Figure 4.—Insuloma of the pancreas of a rat. × 355

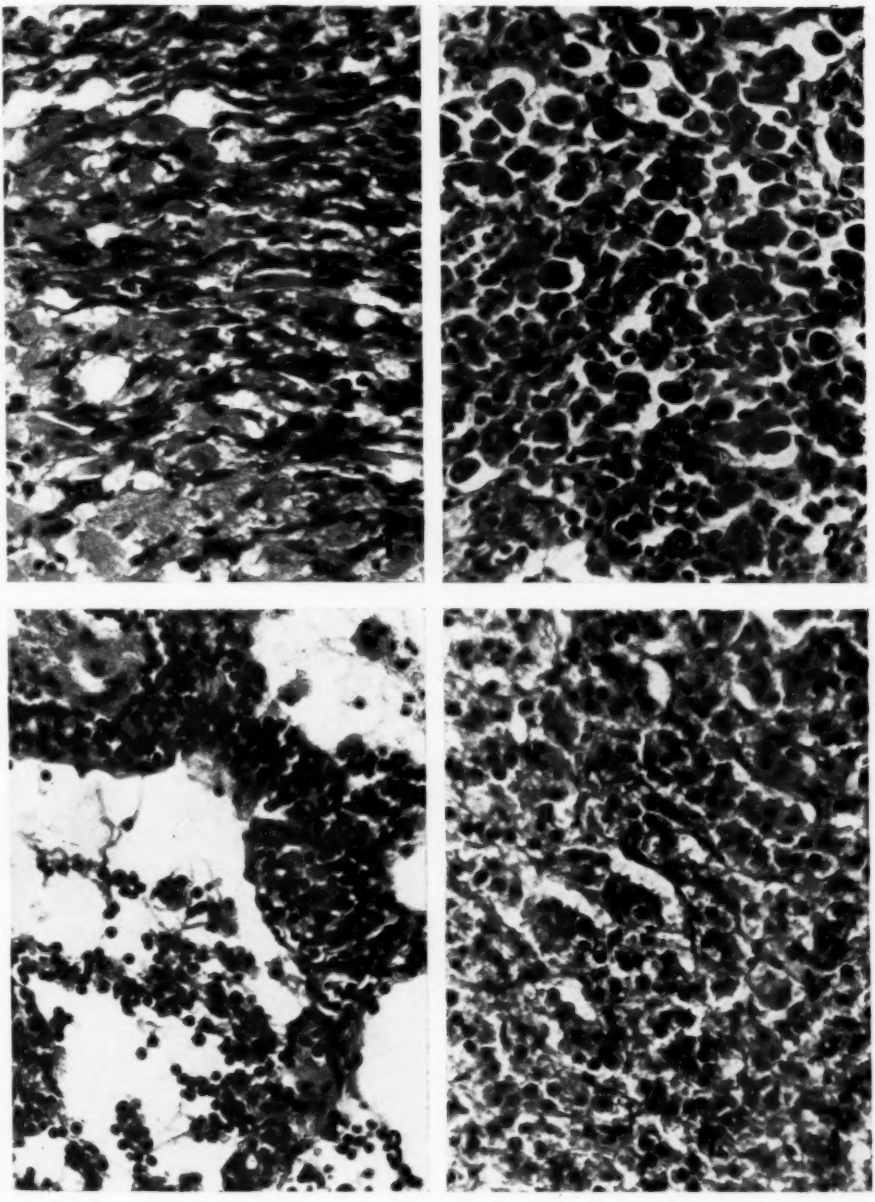

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PLATE 28

FIGURE 5.—Hemangiomatous structures in the adrenal medulla of a rat. × 355

FIGURE 6.—Irregular glandular adenocarcinomatous formations in the marginal portions of an ulcer of the cecum of a rat. × 355

FIGURE 7.—Round-cell sarcoma of the foreleg of a rat, originating apparently from periosteal tissue. × 355

FIGURE 8.—Insuloma of the pancreas of a rat. × 355

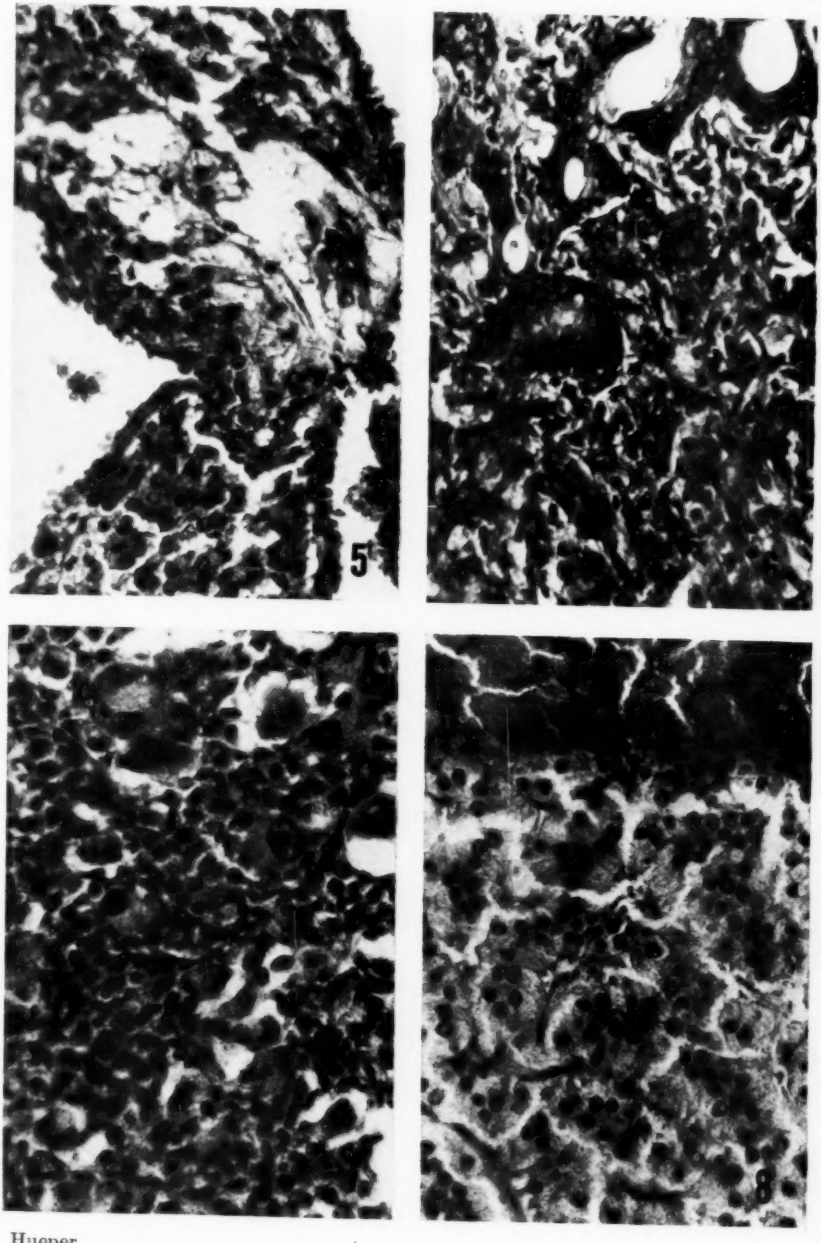
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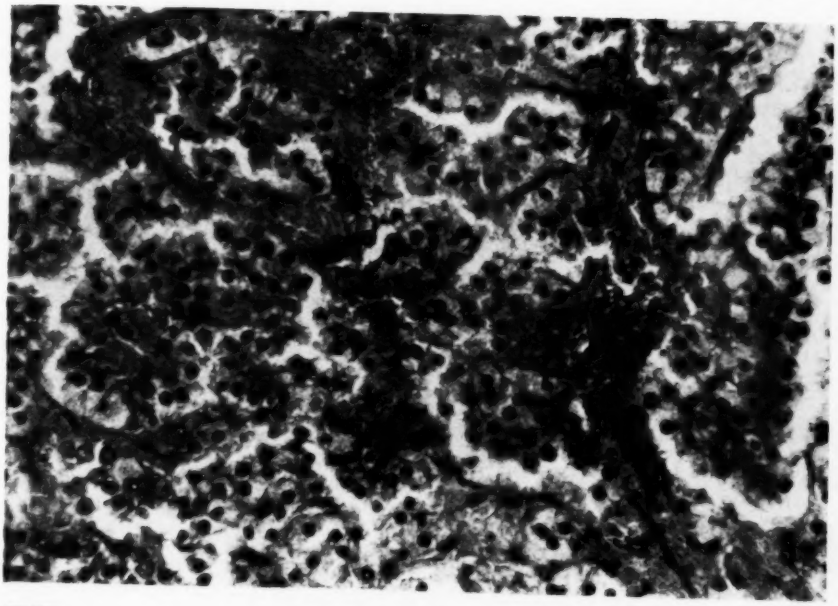
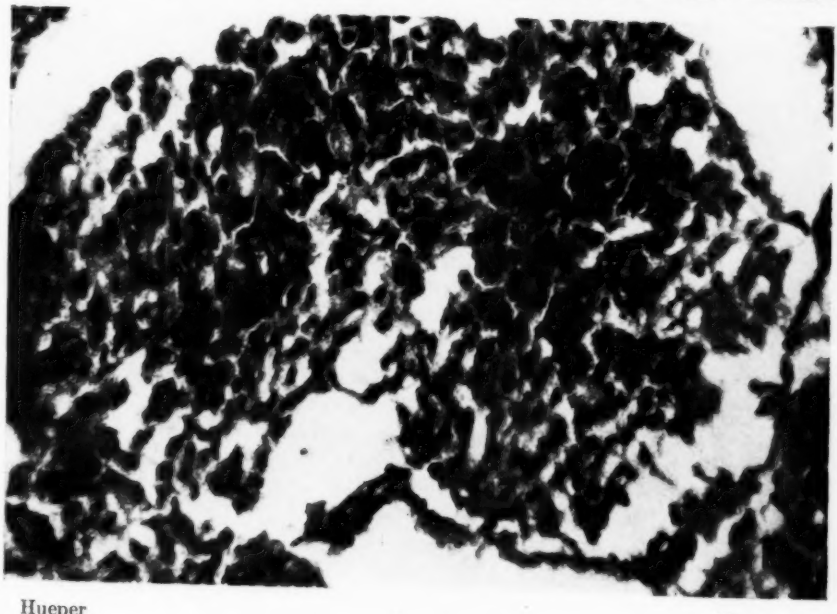
PLATE 29

FIGURE 9.—Insuloma of the pancreas of a rat. × 355

FIGURE 10.—Hemangiosarcoma of the adrenal medulla of a rat. × 355





Hueper

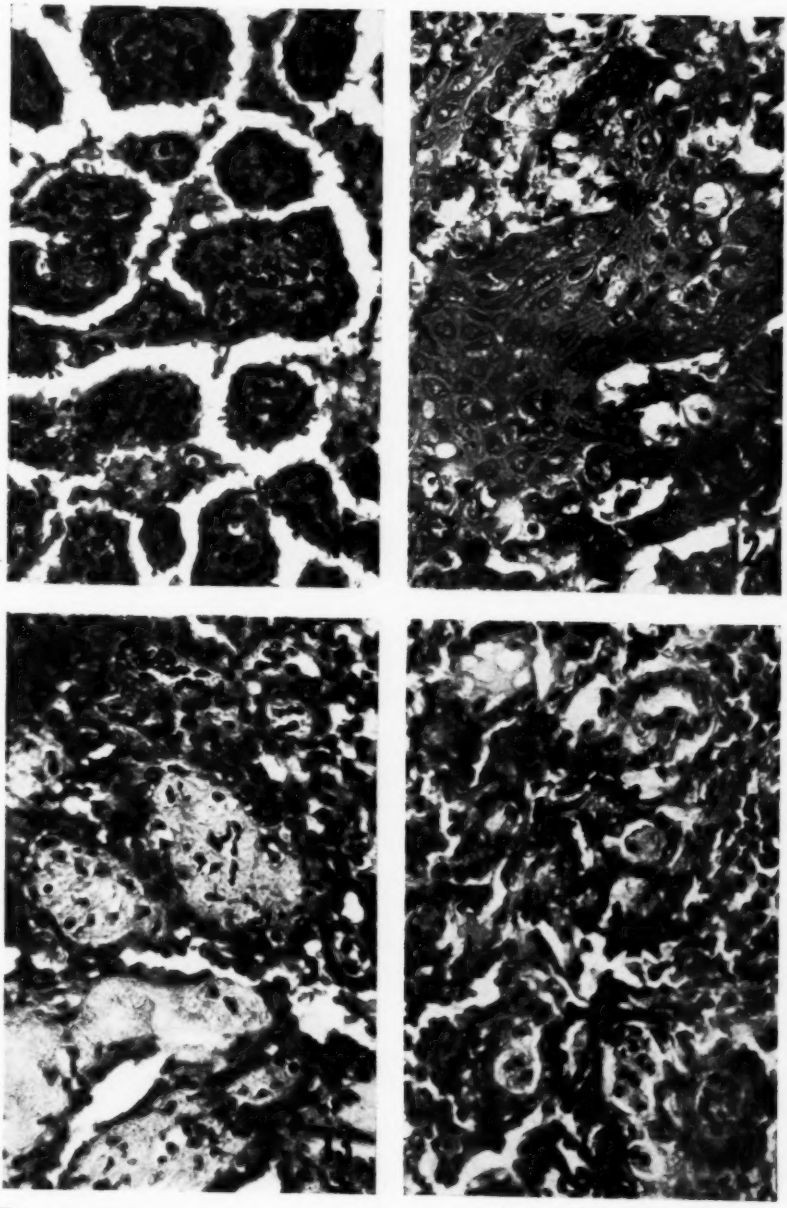
PLATE 30

FIGURE 11.—Papillary adenoma of the lung of a rat. × 355

FIGURE 12.—Papillary adenoma with squamous-cell carcinomatous metaplasia of the lung of a rat. × 355

FIGURE 13.—Islands of mucinous tissue surrounded by small oval and round cells which, in places, form pseudoglandular formations such as are found in synoviomas; tumor of the spermatic cord of a rat. × 355

FIGURE 14.—Densely placed, irregular glandular formations surrounding, as broad cuffs, small and large bronchi, such as are found in peribronchial carcinomatous lymphangitis. × 355



Lack of Carcinogenicity of Lyophilized *Schistosoma* in Mice[1]

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Ferguson (*1*) in 1911, recorded the association of infestation by *Schistosoma haematobium* and carcinoma of the urinary bladder in Egypt. Although this association is usually accepted as having causal relationship (*2*), we are not acquainted with definitive epidemiologic evidence supporting this contention; nor can we find any reports of laboratory studies on the effect of this parasitic infestation upon the occurrence of neoplasms in animals.

Laboratory investigations of the possible carcinogenic effects of bilharziasis are made difficult because such infection is poorly tolerated by mice and rats. The demonstration by Dunning and Curtis (*3*) that peritoneal sarcomas develop in rats following injection of washed, ground-up larvae of *Cysticercus fasciolaris*, the larval form of *Taenia taeniaformis*, as well as following infection by the living organism (*4*), suggested that their method could be applied to the study of possible carcinogenic materials in species of *Schistosoma*.

Experiment 1.—Eggs of *Schistosoma mansoni*, obtained in Egypt in February 1951, were hatched in water. *Biomphalaria boissyi* snails were exposed to miracidia and cercariae subsequently obtained. Mice were infected by immersing their legs in water containing the cercariae. The mice were killed after 4 weeks. Dissection of the liver and the portal circulation yielded living immature worms of both sexes. The worms were washed in distilled water, rapidly frozen, and dried in vacuum.

Male mice of the C3H strain, 3 months old, were used to test the material. The dried worms were moistened with physiological saline and injected through a trocar into the subcutaneous tissue of the mice. Eight mice were injected with 1 worm each, 8 received 6 worms each, and in 8 the dose was from 10 to 16 worms. The mice were maintained in wooden boxes, on Purina dog chow and an unlimited supply of water. They were examined every 2 weeks for palpable tumors at the site of injection, and, upon death, by post-mortem dissection.

Of the 24 mice that were injected, 19 lived for 12 months, 11 were alive at 18 months, and 9 were alive at the termination of the experiment at 21

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[2] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

TABLE 1.—*Lyophilized* S. haematobium *material in strain C3H male mice*

Group	Material	Source	Route injected	Vehicle	Dose (*mg.*)	Mice (*number*)	Survival, in months, after injection						
							3	6	9	12	15	17	20
I	Adults	Gold Coast	SC	Saline	4	10	10	10	9	9	9	9	8 killed at 20 mos.
II	Eggs	Gold Coast	SC	Saline	4, 3*	10	10	10	9	9	9	8	6 killed at 20 mos.
III	Eggs	Gold Coast	IP	Saline	4	10	10	9	9	9	9	6	6 killed at 20 mos.
IV	Adults	Egypt	SC	Saline	4	9	5	5	5	4	4	4 killed at 17 mos.	
V	Eggs	Egypt	SC	Saline	1.5	10	10	7	5	4	1	—	
VI	Eggs	Egypt	SC	Tricaprylin	10	10	10	—	—	—	—	—	
VII	Adults	Egypt	SC	Tricaprylin	6	6	6	6	6	6 killed at 13 mos.			

*Second injection, same site, 3 months after first.

months after injection of the worms. No tumors at the site of injection were elicited. Of the 9 mice killed at the age of 24 months, 3 had hepatomas and 1 had a single pulmonary tumor. These findings parallel closely the reported frequency of these tumors in mice of the C3H strain (*5*).

Experiment 2.—Schistosoma haematobium from the Gold Coast and from Egypt are maintained at the National Microbiological Institute. Eggs were hatched in water. A species of *Bulinus* from Dawa and *B. contortus* snails from Cairo were exposed to the miracidia of the two respective strains. The cercariae were placed on shaved abdominal skin of anesthetized hamsters. The hamsters were killed after 80 to 100 days. Dissection and perfusion of the portal and mesenteric circulation yielded adult worms of both sexes. These were lyophilized and sealed under nitrogen.

The eggs were obtained by digesting the viscera of the hamsters overnight with acid pepsin. The material was then put through a Waring blendor, and the sediment was washed and centrifuged several times. The eggs, with a possible trace of residual tissue, were lyophilized and sealed under nitrogen until they were injected into mice.[3]

The test animals were male mice of the C3H strain, 2 months old. The lyophilized *Schistosoma* material was weighed, suspended in saline or in tricaprylin, and injected subcutaneously through a trocar. In one group of mice the injection was intraperitoneal. Another group received two injections of egg material at an interval of 3 months; all other animals received single injections only. The injected amounts ranged from 1.5 to 10 mg. Table 1 gives details regarding the dose and the survival of mice in each group. The mice were examined periodically for the presence of tumors for 13 to 20 months following injection.

No tumors attributable to the injection of lyophilized *S. haematobium* material were encountered in the animals. Of the 20 mice killed at the termination of the experiment, when they were approximately 22 months old, 5 had single pulmonary tumors and 3 had hepatomas. The frequency of pulmonary tumors was somewhat higher and the frequency of hepatomas was somewhat lower than expected (*5*), but within random distribution considering the small number of animals.

Conclusion

Under the specific and limited conditions of the present experiments, no tumors were elicited in C3H mice with lyophilized worms of *Schistosoma mansoni*, or lyophilized adult worms or eggs of *Schistosoma haematobium*. Extension of studies to actual long-term infestation of laboratory animals with *S. haematobium* appears desirable.

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The Effects of Tyrosine and Phenylalanine on the Synthesis of Pigment in Melanocytes of Embryonic Chick Skin Cultured *In Vitro* [1,2]

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In recent years the view has become widely accepted that the synthesis of melanin pigment in melanocytes of the vertebrate occurs chiefly through the oxidation of the amino acid tyrosine—a reaction catalyzed by the copper-protein enzyme tyrosinase. Evidence for this function of the tyrosine-tyrosinase system is found chiefly in the results of *in vitro* tests employing extracts and homogenates of skin and melanotic tumors, and in histochemical tests designed to disclose the activity of tyrosinase in living and in fixed material. Significant roles in the melanogenic process have been assigned to sulfhydryl groups, *p*H, temperature, redox potential, etc. [Reviewed by Lerner (*1*), Lerner and Fitzpatrick (*2*, *3*), and Rawles (*4*).]

Thus it would appear that the biosynthetic pathway of melanization has been rather well characterized. There is, however, little direct evidence as to the organization of the melanogenic system in the intact cell, the mobilization of the components of this system, and the mechanisms which control the onset and cessation of melanin synthesis. In view of the theoretical and clinical significance attending problems of pigmentation, for example, in melanotic neoplasms, further studies on the synthesis of pigment by living melanocytes seem to be warranted.

In the present study, the effect of tyrosine on pigment synthesis in tissue explants of skin from chick embryos has been examined. Isolates of skin from a pigmented breed of fowl have been maintained *in vitro* for 24 hours on media containing tyrosine and compared as to intensity of pigmentation with similar isolates cultured in the same manner but without added tyrosine. Under these conditions, tyrosine apparently accelerates pigment synthesis. The closely related compound, phenylalanine, seemingly inhibits pigment formation when tested in like manner.

These results confirm a view deduced from various biochemical studies, namely, that the tyrosine-tyrosinase system operates in pigment synthesis

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in living melanocytes. Further, the methods employed in the course of this study offer some promise as a tool for investigating the role of such agents as hormones, various ions, sulfhydryl groups, etc., which may affect the normal course of melanogenesis.

Experimental Procedures

The experiments were carried out chiefly on isolates of dorsal skin obtained from 7½-day-old embryos of the Barred Rock breed of fowl. These isolates were explanted to sterile media contained in watch glasses resting on moist cotton in petri dish culture assemblies, after the methods of Fell and Robison (*5*) and of Spratt (*6*, *7*). Basal media for testing the effects of tyrosine and phenylalanine on pigment synthesis were of two kinds, both devised by Spratt and herein referred to as *glucose-agar* and *yolk-albumen agar*, respectively. In each of these media, support for the explanted skin was provided by a soft agar gel made up in buffered Ringer's solution. The glucose-agar medium contained glucose at a concentration of 1 gm. percent, and the yolk-albumen-agar medium was made up with a saline extract of the content (yolk plus albumen) of the unincubated hen's egg. Details of the preparation of these media are reported by Spratt (*op. cit.*).

For testing the effects of tyrosine and phenylalanine, the amino acids were added during preparation of the agar in appropriate amounts to give the desired final concentrations. The *p*H of both media when freshly prepared was approximately 7.8. Upon incubation, however, they became somewhat more alkaline.

To provide isolates of back skin for culture on these media, embryos of the desired age of incubation were placed in sterile Ringer's solution and dissected with iris scissors and steel forceps. Each embryo provided two "pairs" of explants from the region between shoulder and pelvic levels. The "pairs" comprised contralateral isolates, approximately 2 by 4 mm., excised from either side of the dorsal mid-line. One member of each pair of isolates was explanted to the test medium and the other to the control. Right and left members of the paired isolates were alternated on experimental and control media in order to randomize any possible differences in pigmentation potential between right and left sides of the embryo.

The isolates were oriented on the surface of the medium with the epidermis upward, and the completed cultures placed in the incubator at 37 to 38° C. for 24 hours. They were then fixed *in situ* with Bouin's solution, removed from the surface of the medium, dehydrated, and cleared. Both members of a pair were mounted on one microscope slide under separate cover glasses and subsequently compared as to pigment intensity.

Attempts to obtain objective measurements of pigment content as a basis for comparison were not entirely satisfactory. In isolates which have been cultured, as well as in untreated skin fragments, the pigment cells are often more concentrated in the ectoderm associated with subjacent dermal condensations (*8*)—the anlagen of dermal papillae—and are

more numerous in regions of the explant near the original mid-dorsal line of the embryo. Also, within any one explant, there is considerable variability in the shape and size of the melanocytes and the distribution of their pigment. For these reasons, measurements of optical densities were very difficult and unreliable as a basis for comparing pigment intensities, and counts of differentiated melanocytes were likewise unsatisfactory. Accordingly, in the absence of other methods for making objective comparisons of pigment intensity between isolates grown on different media, comparisons which were somewhat subjective had to be made.

These comparisons were made by a team of three people, consisting of an observer and two assistants. One assistant placed slides bearing the mounted pairs of isolates on the stage of a binocular dissecting microscope in random orientation, so that the observer could not identify either isolate of a pair as to its previous experimental treatment. The observer then compared the isolates as to pigment intensity and reported his results to the other assistant who recorded them. Only the second assistant could identify each tissue with respect to the medium on which it was cultured.

Where no clear-cut distinction in pigment content could be made between isolates of a pair, they were recorded as being of equal pigmentation. Otherwise, one or the other member of the pair was recorded as showing more pigment. At least two, and sometimes three, independent comparisons were made for each set of experimental slides, the results showing very good agreement.

Observations

In preliminary experiments, both of the basal media noted above were examined for their ability to support growth and pigmentation in explants of back skin. On the yolk-albumen-agar medium, isolated pieces of back skin showed an essentially normal growth and differentiation of feather germs and pigment cells for a period of 5 days or more, even when excised from the embryo as early as the fifth day of incubation. With glucose-agar medium, on the other hand, similar isolates could be maintained for only 24 to 48 hours, during which time little if any growth occurred, and feather germs failed to differentiate. Pigment synthesis proceeded for about 24 hours after explantation, but only if donor embryos were of approximately 7½ days of incubation—a stage when appreciable numbers of differentiated melanocytes are already present in the skin.

Morbeck and Saunders (*9*) have reported briefly on the effect of L-tyrosine on pigment synthesis in embryonic skin cultured on yolk-albumen-agar medium. A total of 54 pairs of explants was made in which one member of each pair was cultured on the basal medium, while the other one was placed on similar medium to which tyrosine had been added to give a concentration of 16 mg. percent. In 47 of these cases (87 percent) the isolate cultured with tyrosine produced more pigment in 24 hours than its control. In the remaining cases, the members of a pair were

judged to be of equal pigment intensity. Figure 1 illustrates a typical pair of skin isolates studied in this series of experiments. Differences in pigment intensity of the order shown in this figure are representative of the great majority of cases.

In general, explants grown in medium with tyrosine appeared to have more dendritic melanocytes with darker and more numerous pigment granules than their controls. This impression of difference could not be expressed quantitatively, however, because of the difficulties outlined above.

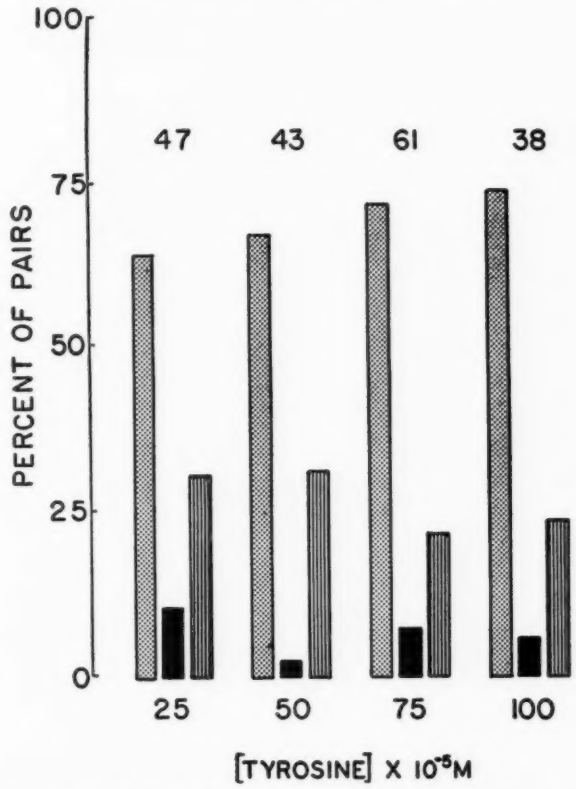
Conceivably, in these experiments, the availability of tyrosine affected directly the rate of pigment synthesis, possibly by making available for utilization in melanin synthesis more of the amino acid than is normally present in embryonic skin. It was considered desirable, however, to examine this possibility further by culturing the explants on media of known chemical composition and under circumstances which do not permit appreciable growth and differentiation of the skin. Accordingly, glucose-agar medium was employed in subsequent experiments, which form the chief basis of this report. Donor embryos were incubated for 7½ days prior to explanting the skin isolates in order to assure that melanin synthesis would take place in the cultures.

In the first series of experiments, 189 pairs of skin isolates were studied. One member of each pair was explanted to basal medium, while the contralateral isolates were placed on media containing L-tyrosine at concentrations ranging from 25×10^{-5} to 100×10^{-5} molar. After culturing for 24 hours, the paired skin fragments were fixed, mounted, and then compared as to pigment intensity. The results are graphed in text-figure 1.

It is evident from this graph that the frequency with which skin isolates grown on media containing tyrosine show a greater intensity of pigmentation than their contralateral isolates cultured on basal medium is quite high, *i.e.*, 64 to 74 percent of the cases. In contrast, the control explants showed more pigment in only 2 to 7 percent of the pairs; and in 21 to 29 percent of the cases, little or no difference in pigment content between members of a pair was apparent. It is worthy of note that at the higher concentrations of tyrosine, decisions as to comparative pigment intensity between members of a pair were generally easier to make.

In a second group of experiments, 304 pairs of skin explants were made. One member of each pair was cultured on medium containing L-tyrosine at concentrations ranging from 25×10^{-5} to 100×10^{-5} molar; the other member was placed on medium containing L-phenylalanine at the same molar concentration as that of the tyrosine. Phenylalanine is apparently not immediately oxidized to melanin, and, since it differs from tyrosine only in that it lacks an –OH group, it should provide controls for osmotic pressure, available amino groups, nutrient energy of the medium, etc.

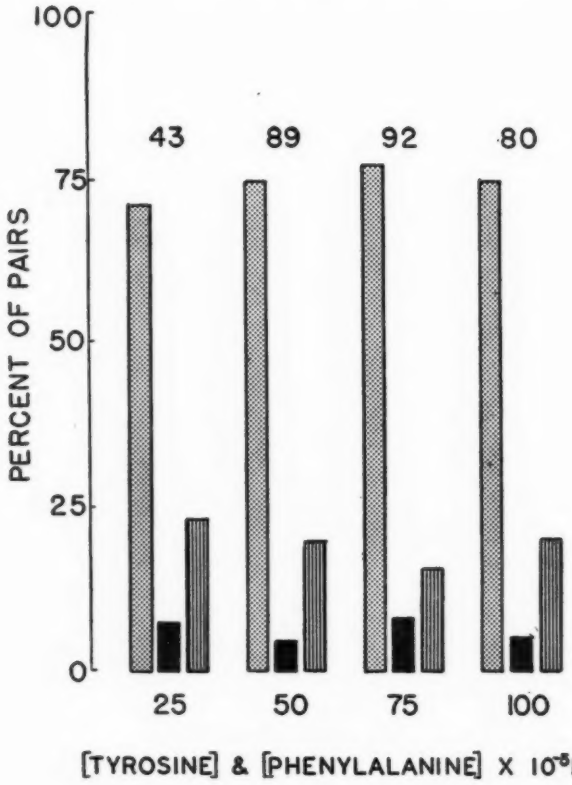
The results of this series of experiments are graphed in text-figure 2. They show that, at all concentrations of the amino acids tested, those isolates supplied with exogenous tyrosine were more intensely pigmented



TEXT-FIGURE 1.—The effect of adding tyrosine to the basal medium on the synthesis of pigment by isolates of embryonic chick skin *in vitro*. *Stippled bars:* the percent of cases in which explants showed more intense pigmentation on medium containing tyrosine. *Solid bars:* the percent of cases in which the explant to basal medium produced more pigment. *Vertically hatched bars:* the percent of cases in which both explants were equally pigmented. Total number of pairs tested at each concentration of tyrosine is shown at the top of the figure.

than their contralateral isolates in 69 to 77 percent of the cases. In only about 5 to 8 percent of the pairs of explants, the isolate cultured on the medium with phenylalanine showed more pigment; and in 15 to 23 percent of the cases, both members of each pair were judged as being equally pigmented.

These results suggest rather strongly that the presence of tyrosine in the medium promotes melanogenesis in differentiating pigment cells. As a further control, however, another series of experiments was undertaken in which isolates of embryonic skin, cultured on basal medium alone for 24 hours, were compared as to pigment intensity with their contralateral isolates which were cultured on media containing L-phenylalanine at concentrations ranging from 25×10^{-5} to 100×10^{-5} molar. The results,



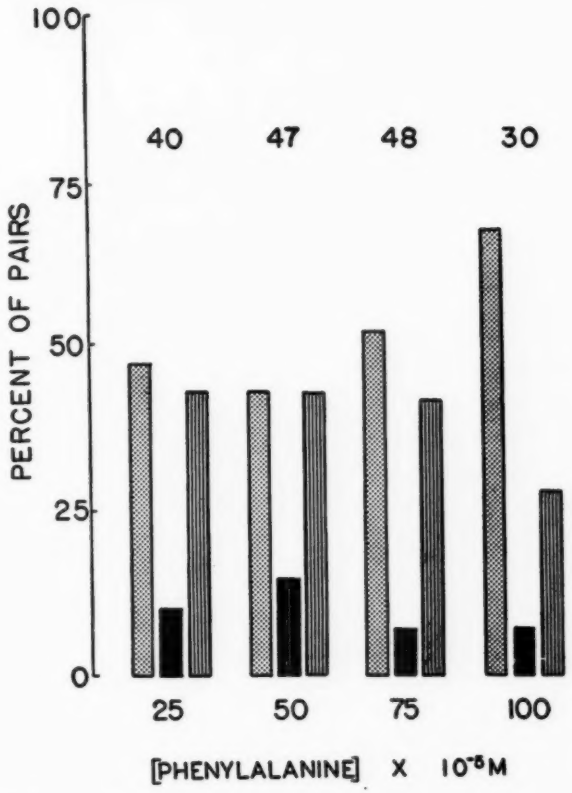
TEXT-FIGURE 2.—The production of pigment by skin from chick embryos on media containing tyrosine, as compared to similar cultures with phenylalanine at equal concentrations. *Stippled bars:* the percent of cases in which the explant to medium containing tyrosine showed more pigment. *Solid bars:* the percent of cases showing more pigment in the explant to medium containing phenylalanine. *Vertically hatched bars:* the percent of cases in which both explants of a pair showed equal pigment intensity. The total number of pairs tested at each concentration of tyrosine and phenylalanine is shown at the top of the figure.

presented in text-figure 3, showed that explants receiving phenylalanine produced more pigment than their contralateral isolates in only 7 to 15 percent of the cases. In contrast, explants to basal medium alone produced more pigment in 42 to 66 percent of the cases, while both isolates were equally pigmented in 26 to 42 percent of the pairs. Apparently, therefore, phenylalanine inhibits to some extent the synthesis of melanin in skin from embryonic chicks under the conditions of these tests, most notably at the higher concentrations.

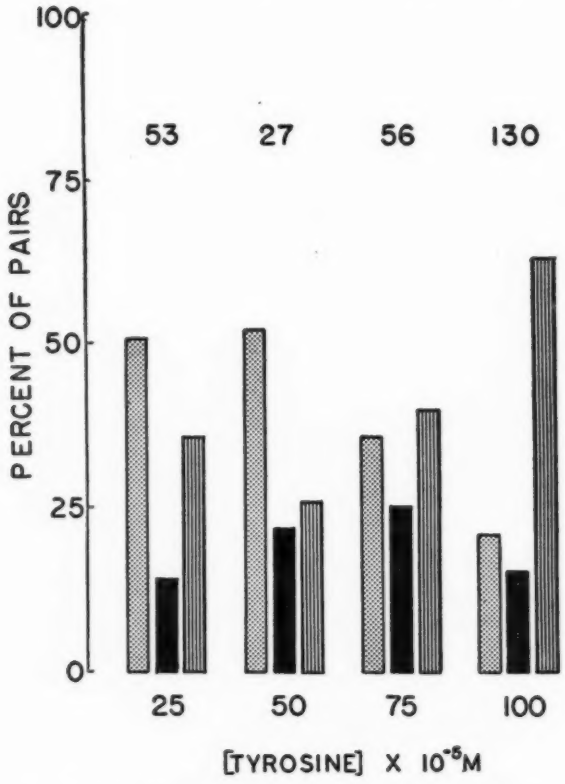
Subsequently, experiments were undertaken to determine whether the apparent inhibitory effects of phenylalanine on the melanogenic process might be prevented by tyrosine. An extensive series of tests showed

that phenylalanine inhibits only when its concentration in the culture medium is considerably in excess of that of tyrosine. For these experiments, isolated skin pairs were explanted as before. Each member of a pair was cultured on medium containing the same concentration of L-tyrosine, the range being from 25×10^{-5} to 100×10^{-5} molar. In addition, L-phenylalanine was supplied to one member of each pair in a concentration of 100×10^{-5} molar. The results of these tests are presented in text-figure 4.

Under these conditions, as the figure illustrates, the member of a pair having phenylalanine in the medium at high concentration (100×10^{-5} *M*) and tyrosine at low concentration (25 and 50×10^{-5} *M*) generally showed



TEXT-FIGURE 3.—The inhibition of pigment synthesis in isolates of skin from chick embryos cultured with varying concentrations of phenylalanine. *Stippled bars:* the percent of cases in which the explant to basal medium produced more pigment. *Solid bars:* the percent of cases in which the member of a pair cultured with phenylalanine produced more pigment. *Vertically hatched bars:* the percent of cases in which both members of the explanted pair developed equal pigmentation. The total number of pairs tested at each concentration of phenylalanine is shown at the top of the figure.



TEXT-FIGURE 4.—The production of pigment by skin from chick embryos in cultures containing tyrosine as compared to similar cultures containing both tyrosine and phenylalanine. *Stippled bars:* the percent of cases showing more pigment in the explant to basal medium plus tyrosine. *Solid bars:* the percent of cases in which the explant to medium containing both tyrosine and phenylalanine produced more pigment. *Vertically hatched bars:* the percent of cases in which both explants developed equal pigmentation. The total number of cases tested at each concentration of tyrosine is shown at the top of the figure.

less pigment than its contralateral explant that received tyrosine alone. In contrast, however, explants with phenylalanine (100×10^{-5} *M*) and tyrosine (75 and 100×10^{-5} *M*), both at high concentration, usually produced just as much pigment as their contralateral explants receiving only high concentrations of tyrosine. Apparently, therefore, the inhibition of melanogenesis by phenylalanine may be prevented or reversed by tyrosine.

Discussion

The experiments reported here show that isolates of skin from embryonic chicks cultured in media containing L-tyrosine are, in a high percentage of the cases, more intensely pigmented after 24 hours *in vitro* than are

similar isolates cultured in media without this amino acid. In contrast, explants to media containing L-phenylalanine only infrequently show more pigment than those cultured in basal medium; characteristically the latter show more pigment, particularly when high concentrations of phenylalanine are employed. Apparently, therefore, the synthesis of melanin by embryonic melanocytes of the chick is augmented by tyrosine, whereas phenylalanine tends to inhibit the process. Moreover, this inhibitory effect on melanogenesis which is shown by phenylalanine may be reversed by tyrosine, since explants to media in which tyrosine and phenylalanine are combined in suitable amounts are usually as intensely pigmented as those cultured in media to which tyrosine alone has been added.

In view of the considerable body of evidence that melanin in vertebrate pigment cells is derived from the enzymatic oxidation of tyrosine (*1–3*), it is reasonable to interpret the augmentation of pigment production in chick skin cultures by tyrosine as the result of increasing the concentration of substrate available to the enzyme tyrosinase in differentiating melanoblasts. Presumably cells in which tyrosinase activity has differentiated have sufficient enzyme to oxidize more tyrosine than is normally available for pigment synthesis. According to this interpretation, tyrosine of exogenous origin, if able to enter the melanoblast, would be synthesized to melanin during the incubation of the cultures. Preliminary studies (unpublished) of the distribution of uniformly labeled (C^{14}) tyrosine in melanocytes of similar cultures have confirmed this view. Radioautographs of these cultures show that melanocytes which differentiate during exposure of the skin to medium containing tyrosine labeled with C^{14} are strongly radioactive, while other skin cells show little or no activity.

In this connection, it is of interest to note that Markert (*10*) has also recently demonstrated by means of radioautographs that uniformly labeled (C^{14}) tyrosine is synthesized to melanin in melanocytes of embryonic chick skin cultured in standard (hanging-drop) plasma-clot preparations. When tyrosine labeled only in the side chain was supplied to his cultures, however, differential radioactivity was not observed in the melanocytes. Thus Markert's results may cast some doubt on the usually accepted scheme of melanin synthesis (*2*), but they do confirm the concept of a rather direct participation of tyrosine in the melanogenic process.

The present experiments also raise a question concerning the role of phenylalanine in melanin synthesis. Since phenylalanine is apparently oxidized enzymatically to tyrosine in a number of vertebrate tissues (*1*), it would seem possible that it might augment melanin synthesis in isolates of skin from chick embryos in somewhat the same manner as tyrosine. As the results show, however, exogenous phenylalanine does not promote melanogenesis under the conditions of these experiments, but rather, seems to inhibit the process. On the assumption that phenylalanine is taken up by the differentiating melanocyte, it is reasonable to suggest that it may inhibit melanin synthesis by competing with intracellular tyrosine for combination with tyrosinase, thus displacing the normal sub-

strate of the enzyme. This interpretation is suggested by the similarity of tyrosine and phenylalanine in structural configuration and by the fact that phenylalanine inhibition of melanogenesis may be prevented by the simultaneous presence in the culture medium of adequate amounts of tyrosine. It is also in accord with current experimental results which show that other compounds structurally related to tyrosine (3-fluorotyrosine and L-3,5-diiodotyrosine) inhibit melanogenesis in skin isolates cultured *in vitro* in a manner similar to phenylalanine, and this inhibition is likewise reversed by tyrosine [*cf.* Woolley (*11*) for an analysis of metabolic competition between structurally related compounds; also, McElroy (*12*)].

Obviously, a more suitable method for examining the possibility that phenylalanine competitively inhibits the melanogenic reaction should employ data for the rate of melanin synthesis by pigment cells in culture supplied with various concentrations of phenylalanine alone and in combination with varying concentrations of tyrosine. To provide a basis for calculating reaction rates, current experiments are directed toward development of a method for the quantitative assay of pigment synthesized by embryonic melanocytes during incubation of the cultures.

The differentiation of melanoblasts in skin and feathers of the fowl and the kind and amount of pigment they synthesize is determined to a large extent by the characteristics of the epidermal tissue environment in which the melanoblasts are found (*4*, *13*, *14*). In the present study, it has been observed that skin isolates explanted to glucose-agar medium prior to 7 or 7½ days of incubation fail to form feather germs, and their melanoblasts do not synthesize appreciable quantities of pigment, even in the presence of supplementary tyrosine. In explants to this medium from slightly older embryos, however, pigment is synthesized, the more so if tyrosine is added, but feather germs do not differentiate. It would appear, therefore, that conditions for the differentiation of melanocytes and for their utilization of tyrosine in pigment synthesis are provided only in an epidermis of a certain stage of maturation. Skin isolates explanted to glucose-agar medium prior to 7 or 7½ days of incubation do not achieve this stage.

With yolk-albumen-agar medium, on the other hand, explants of embryonic skin grow and differentiate in a relatively normal manner even when explanted at very young stages. This medium is thus adequate to support the progressive maturation of the epidermis, which then provides a substratum in which embryonic melanocytes may differentiate and synthesize pigment.

The problem remains, however, to correlate the organizational features of the maturing skin and feather germs with the differentiation of tyrosinase or related enzyme activities in the melanoblasts, and with the distribution of tyrosine or other substrates on which the enzymes act. In this connection, it is of interest that a number of factors, such as copper, sulfhydryl groups, redox potential, electrolyte concentration, etc., have been shown to affect the melanogenic reaction *in vitro*, and to some extent *in vivo* [(*3*); see also (*15*)]. It would seem that the method used in the present study might be employed to explore the possibility that these and

other factors, such as hormones, may participate in the normal differentiation of the melanogenic system in embryonic skin.

Summary

The effects of L-tyrosine and L-phenylalanine on the synthesis of pigment by melanocytes of the chick have been examined in isolates of embryonic dorsal skin cultured *in vitro*. Explants cultured for 24 hours in a medium containing tyrosine are, in a high percentage of the cases, more intensely pigmented than similar explants to media without tyrosine, thus suggesting that tyrosine is utilized by embryonic melanocytes of the chick for pigment synthesis.

Under similar conditions, skin isolates explanted to media with phenylalanine generally produce less pigment than do isolates cultured without phenylalanine. Phenylalanine thus apparently inhibits the melanogenic process in skin from chick embryos. This inhibition, which may be reversed by the addition of tyrosine to the medium, is possibly of a competitive type.

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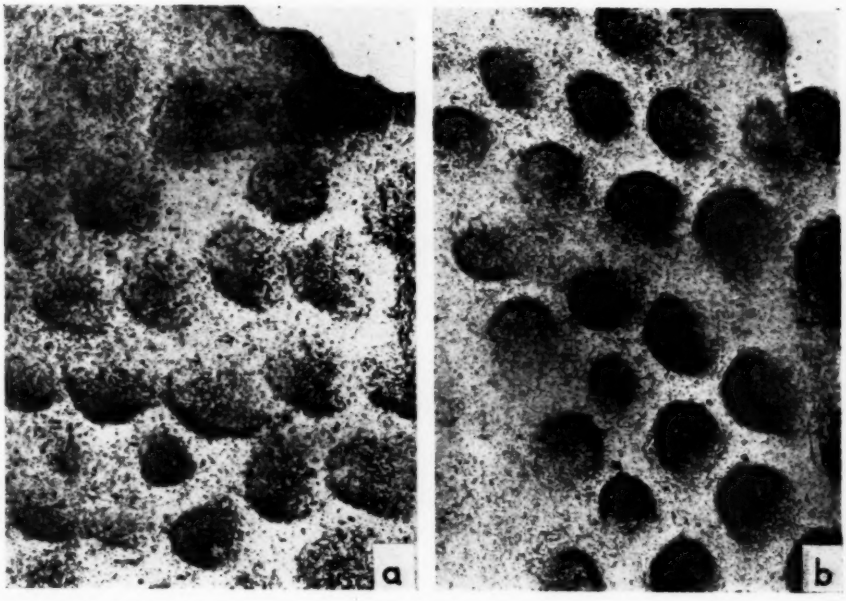
PLATE 31

FIGURE 1.—Contralateral isolates of dorsal skin from a 7½-day-old Barred Rock chick embryo cultured for 24 hours on yolk-albumen-agar medium: *a*, without tyrosine; *b*, with tyrosine.



Saunders *et al.*

352471—55——11

Tumors Induced by 2-Acetylaminofluorene in Buffalo-Strain Rats Ingesting Different Levels of Riboflavine[1,2]

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Riboflavine deprivation in the rat has frequently been reported to enhance hepatoma induction by the liver-specific, distant-acting carcinogen 4-dimethylaminoazobenzene. This effect of riboflavine prompted an investigation to ascertain whether or not different levels of dietary riboflavine would influence the induction of any of several types of tumors by the non-organ-specific, distant-acting carcinogen 2-acetylaminofluorene (2-AAF). Metabolism studies indicated that a deficiency of dietary riboflavine delayed the absorption of 2-AAF-9-C^{14}, although it had no effect on the distribution or protein binding of radioactivity in the organs or tissues tested (*1*). A larger proportion of water-soluble metabolites, however, such as glucuronides of hydroxylated compounds and smaller amounts of ether-soluble compounds, *e.g.*, 2-aminofluorene, 2-acetylaminofluorene, and their monohydroxylated derivatives, appeared to be excreted by riboflavine-deficient rats (*1*).

The increased water-soluble and decreased ether-soluble metabolites produced by the extremely riboflavine-deficient animal during acute experiments may possibly be explained by the delayed absorption of 2-AAF, which could lower 2-AAF or its metabolite concentration in the organs or tissues involved in degrading the carcinogen. If similar metabolic conditions exist during chronic experiments, one might expect different levels of riboflavine intake to alter either the type or number of tumors induced in rats by 2-AAF. One insurmountable obstacle in such carcinogenic studies with severely depleted rats, however, is their inability to survive prolonged periods of 2-AAF administration. Sufficient riboflavine must be provided to permit reasonable survival.

Harris (*2*) studied the carcinogenic activity of ingested 2-AAF in male and female Wistar-strain rats ingesting a high-riboflavine, high-protein diet on the one hand, and a low-riboflavine, low-protein diet on the other. His data indicate no effect from the addition of 0.8 mg. percent of dietary riboflavine on the development of liver tumors in male and female rats, but in the female addition of only 0.2 mg. percent dietary riboflavine may

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[2] Presented, in part, before the 128th meeting of the American Chemical Society, Minneapolis, Minn., Sept. 12–16, 1955.

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[4] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

352471—55——12

have retarded liver-tumor development. No information was presented on the 2-AAF intake in any of Harris' experiments, and if lowered food intake occurred in the low-riboflavine-diet group, the decreased 2-AAF intake resulting therefrom could possibly have influenced the carcinogenic response.

Engel and Copeland (*3*) state that varying the riboflavine content of their basal diet (containing 10.6% casein) from 1 to 100 μg. per gram failed to influence tumor incidence or tumor-induction time in female rats of the A.E.S. strain ingesting 2-AAF. The details of their experiments, however, have not been published. The experiments described herein were designed to maintain a constant intake of the carcinogen during the experimental period when rats were ingesting diets containing low and high levels of riboflavine. The influence of variations in quantity of riboflavine intake was noted for all induced tumors, but especially for mammary tumors.

Methods

Three groups, each consisting of 18 Buffalo-strain female rats, 3 to 4 months of age, were placed on purified semisynthetic diets containing 24 per cent of vitamin-free casein[5] and 1, 2, and 10 μg. of riboflavine per gm. of diet in experiments 1, 2, and 10, respectively. Each rat was given 6 mg. of 2-AAF per 100 gm. body weight weekly. The carcinogen, dissolved in warm coconut oil, was administered by stomach tube twice a week for the first 3 weeks and once a week thereafter for the duration of the experiment. This change in dosage schedule was made in an effort to keep the animals alive for a longer period of time. During any given experimental period all rats received the same amount of carcinogen under identical conditions of administration. The longer an animal survived, however, the greater was its total intake of 2-AAF.

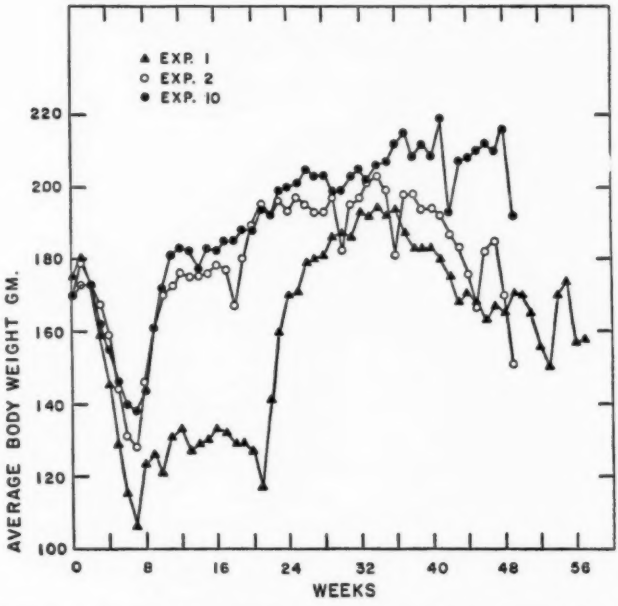
Results and Discussion

Owing to the high toxicity of 2-AAF to rats receiving 1 and 2 μg. of riboflavine per gram of diet, more than 60 percent of the animals failed to survive beyond 8 weeks, a period normally too short for 2-AAF to induce tumors. Only one third of the animals in experiments 1 and 2 survived 20 or more weeks—a period long enough for the earliest tumors to develop. However, 60 percent of the animals receiving 10 μg. of riboflavine per gram of food (expt. 10) survived 28 or more weeks. Riboflavine at the higher level, therefore, definitely prolonged survival of Buffalo-strain female rats given 2-AAF.

The average body weight of the animals in all 3 experiments decreased 40 to 75 grams (text-fig. 1) during the first 7 weeks of the experiment. Animals in experiment 1 regained very little weight until after the 21st week; between the 21st and 31st weeks they regained all the weight previously lost. Weight loss again occurred after the 36th week in experi-

[5] Composition of diet by weight.—Vitamin-free casein 240, cerelose 527.5, salt mixture No. 185a (*4*) 30, cod-liver oil 10, lard 192, choline chloride 0.5, and a vitamin mixture containing per gram of diet the following amounts of vitamins in μg.: folacin 1, biotin 0.1, thiamine 3, pyridoxine 2, calcium pantothenate 14, niacin 20.

ment 1. The average weight of animals in experiments 2 and 10 was essentially parallel, and after the initial large loss the average starting weight in both experiments was recovered between the 7th and 12th weeks. No explanation is apparent for these body-weight changes since the dosage of 2-AAF was the same per unit weight in all 3 experiments. One possible explanation could be that a critical level of riboflavine occurs between 1 and 2 μg. per gram of food in this strain on this particular diet when the dosage of 2-AAF is administered as in these experiments. Survival was not affected but body weight was changed. The tumor incidence is presented in table 1.



TEXT-FIGURE 1.—Average weekly body weights of Buffalo rats surviving 5 or more months.

Although the number of rats autopsied was small, there did not appear to be any difference in the induction of hepatomas between the 1 and 2 μg. and the 10 μg. levels of riboflavine intake. Combining all the data in the low-riboflavine experiments 1 and 2 gave values for induced breast and acoustic-duct tumors of approximately half those which occurred at the 10 μg. level of riboflavine intake in experiment 10.

Thirty-one tumors were observed in 11 rats autopsied after receiving the high-riboflavine diet and 13 tumors in 11 rats autopsied after receiving the 1 and 2 μg. levels of riboflavine. The average duration of the dosage for the former was 8.8 months and the latter 9.9 months (table 2). The reason for the striking difference is that in the above figures on low levels of riboflavine, 4 rats did not develop any tumors and only 2 animals

TABLE 1.—*Tumors induced by 2-AAF* in Buffalo-strain female rats ingesting diets variable in riboflavine*

Expt. No.	Rats autopsied (*number*)	Dietary level of riboflavine (*μg./gm.*)	Average length of experiment (*months*)	Tumors			
				Hepatoma	Adenocarcinoma of the breast	Squamous-cell carcinoma of the ear duct	Other
1	6	1	11	2†	0	4	0
2	5	2	8	1	5‡	1	0
10	11	10	9	4	12§	9	7‖

*2-AAF administered by stomach tube twice weekly for first 3 weeks, once weekly thereafter.

† If more than one hepatoma arose in the same liver, they were counted only once because of the difficulty in obtaining an exact count of all multiple hepatomas. All other tumors were counted as distinct tumors and the total number was recorded.

‡ Two rats, each with 1 breast tumor; 1 rat with 3 tumors.

§ Three rats, each with 1 breast tumor; 3 rats, each with 2; 1 rat with 3 tumors.

‖ One adrenal-cortical nodule.
One fallopian-tube carcinoma.
Three small bowel adenocarcinomas.
One uterine sarcoma.
One pancreas (gross, large tumor mass in region of pancreas. No microscopic diagnosis made because of post-mortem degeneration).

developed 3 tumors, whereas, at the higher level of riboflavine intake, all animals autopsied developed at least 1 tumor and 2 animals each developed 6 tumors. Multiple hepatomas, in this analysis, were considered as single tumors. The differences in tumor incidence between low- and high-riboflavine intake could not be ascribed to longer survival of animals on the higher intake of riboflavine nor to a greater intake of the carcinogen. Even though the number of animals coming to autopsy was small, a striking effect of riboflavine in promoting tumor induction in Buffalo-strain female rats was obtained. At the low levels of riboflavine intake, no tumors were induced at other sites than the liver; but in the high-riboflavine experiment, 7 were found, although 2 of the 7 were

TABLE 2.—*Distribution of multiple tumors in rats given 2-AAF at high and low levels of riboflavine intake*

Expt. No.	Number of tumors per rat	Number of rats	Length of experiment (*months*)
10	1	3	9, 9, and 9
	2	4	6, 8, 9, and 10
	3	1	11
	5	1	9
	6	2	8 and 9
		11	
1 and 2	0	4	6, 9, 10, and 12
	1	3	6, 10, and 12
	2	2	11 and 12
	3	2	8 and 13
		11	

not diagnosed microscopically because of post-mortem degeneration. The incidence of tumors at "other" sites in the high-riboflavine-diet group was sufficiently higher to be significant. These experiments, as indicated above, were conducted in such a way that the amount of carcinogen each rat received was not influenced by food intake, since 2-AAF was given by stomach tube, and the amount administered was based on the weekly weight of the animal. The rats surviving for the longer periods did get more 2-AAF, yet they did not develop more tumors.

The recovery of body weight in experiment 1, 20 weeks after the experiment was started, may indicate that the animals had become adapted to survival on a lower riboflavine intake in spite of the toxic effect of the administered 2-AAF. Additional studies on the role of riboflavine in tumor induction by 2-AAF appear to be needed. It is concluded from the results obtained under these experimental conditions that riboflavine does not protect rats from the induction of tumors by 2-AAF, thus differing from the protective action of riboflavine on 4-dimethylaminoazobenzene carcinogenesis.

Summary

Eleven Buffalo-strain female rats were given a constant amount of 2-AAF weekly and fed a diet containing 1 and 2 μg. of added riboflavine per gram of food in an otherwise riboflavine-deficient diet. They demonstrated, when autopsied, a total of 3 liver tumors, 5 breast tumors, and 5 acoustic-duct tumors but no tumors at other sites. Eleven female rats receiving the same basal diet, containing 10 μg. of added riboflavine per gram of food, manifested, when autopsied, 4 liver tumors, 12 breast tumors, 9 acoustic-duct tumors, and 7 tumors at other sites—3 of which were in the small intestine. The data suggest that the diet higher in riboflavine given to Buffalo-strain female rats receiving a constant amount of 2-acetylaminofluorene weekly by stomach tube promoted mammary tumors, acoustic duct tumors, and tumors at other sites under the conditions imposed, but had no apparent effect on the incidence of liver tumors.

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Neoplasms in the C3H Strain and in F_1 Hybrid Mice of Two Crosses Following Introduction of Extracts and Filtrates of Leukemic Tissues[1]

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It has been reported by Gross (*1–5*) that leukemia and so-called salivary gland carcinomas were induced in C3H strain mice, of 2 different sublines, following the subcutaneous inoculation of young mice, particularly those from the high-leukemic AK-n strain, with extracts, centrifugates, and filtrates of leukemic tissues.

It is the purpose of this report to summarize our observations to date on nearly 500 mice receiving inoculations, within 24 hours of birth, of extracts, centrifugates, and filtrates of leukemic tissues which were obtained from several high-leukemic strains of mice. Although the incidence of leukemia in our test mice has not been increased, several interesting observations have been made. Particular attention will be given to the induction of pleomorphic tumors of the parotid gland, to attempts to transplant these neoplasms, and to a detailed morphologic description of these tumors. In a small group of F_1 mice, obtained by crossing 2 different sublines of the C3H strain, a high incidence of subcutaneous neoplasms was found in mice in which parotid-gland tumors were also frequently observed. A preliminary report of some of these findings has been given (*6*).

Materials and Methods

Mice.—The test mice used throughout this work were either of our own subline, $C3H_f$/Lw (*7*), or of the Heston subline, $C3H_f$/He (*8*). Our subline was developed from a pair of C3H mice obtained in 1951 from Dr. W. E. Heston, who, in turn, obtained his breeding nucleus from Dr. H. B. Andervont in 1941. Both sublines used have been freed of the mammary tumor milk-agent. Only pedigreed mice of the F-10 to F-15 generation of the $C3H_f$/Lw subline and of the F-20 to F-25 of the $C3H_f$/He were used.

The incidence of leukemia[3] is low in the sublines of C3H mice used in this study. We have previously observed in a sample of our subline less

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[3] The term "leukemia" is used, as is customary in discussing the condition in mice, to refer to neoplastic conditions of the blood-forming organs and the reticuloendothelial system. It does not imply that neoplastic cells are always present in the peripheral blood.

than 1 percent leukemia occurring late in life. On the other hand, Dunn has observed 8.4 percent of reticular neoplasms in a large sample of the He subline after 18 months of age. Less than 4 percent were lymphocytic (*9*).

Since no differences in the incidence of neoplasms were detected in this study between these 2 closely related sublines, they will be referred to hereafter as $C3H_f$ mice.

A few observations have been made using another subline of the C3H strain developed by Dr. F. H. J. Figge (*10*) and herein designated as C3H/Fg. This line was obtained originally from Dr. L. C. Strong in 1941. Our nucleus was obtained from Dr. Figge in 1953 at the F-88 generation. We have observed a 30 percent incidence of leukemia, principally lymphocytic, in our colony, although a much higher incidence has been reported by Figge (*10*).

In addition to strain C3H mice, 2 sets of hybrid mice have been used in this study: $(C3H_f \times AKR)F_1$, obtained by crossing ♀$C3H_f$/Lw × ♂AKR/Lw mice; and $(C3H_f \times C3H/Fg)F_1$, obtained by crossing ♀$C3H_f$/Lw × ♂C3H/Fg mice. The incidence of leukemia, predominantly lymphocytic, in $(C3H_f \times AKR)F_1$ mice is approximately 60 percent and the average age at death of leukemics, 15 months. As yet a large enough sample of F_1 mice of the other cross has not been observed for purposes of determining the incidence of leukemia.

Leukemic materials.—Histologically diagnosed leukemic tissues were obtained from pedigreed mice of 3 high-leukemic strains: C58/Lw (*11*), generations F-85 to F-93; AKR/Lw (*11*, *12*), generations F-42 to F-49; and from the Figge subline, F-90 to F-92, of the C3H strain (C3H/Fg) previously described. Spontaneous and transplanted lymphocytic neoplasms principally from the AKR strain were used. The incidence of leukemia in our subline of the AKR strain is 92 percent and death from leukemia occurs at an average age of 8.0 months. A similarly high incidence of leukemia (90 percent) is found in the C58 strain with death of leukemics occurring at 10.0 months.

Several different methods were used for the preparation of leukemic tissues. Spleen, liver, thymus, and superficial and internal lymph nodes infiltrated by neoplastic cells were taken from mice with spontaneously occurring leukemias. In the case of transplantable leukemias (subcutaneous transfer), the mass of neoplastic tissue, the liver, and the spleen were used; approximately 2 grams of tissue were available from each leukemic mouse. Histologic diagnosis of leukemia was made on all tissues used in this study. These were lymphocytic neoplasms, the morphologic form most frequently found in the mouse, unless otherwise stated in the text or tables.

Method I: Animals were sacrificed by cervical dislocation. Tissues were removed aseptically and ground with Locke's solution in a Ten Broeck homogenizer. A 20-percent cell suspension was used for inoculations.

Method II: The material prepared as in *method I* was centrifuged for 15 minutes at 3,000 r.p.m. in an Aloe angle centrifuge. The upper third of the supernatant fluid was used for injections.

Method IIIA: The supernatant fluid obtained from *method II* was filtered through a tested Berkefeld candle, type N porosity.

Method IIIB: Filtration was accomplished through a Selas #03 filter. All filters were tested with *Escherichia coli* before and during filtration under a vacuum of 20 inches of mercury pressure.

Method IVA: Leukemic cell suspensions were prepared from the same tissues but were ground by the use of a cold mortar and pestle in chilled Locke's solution. The suspensions were centrifuged at 3,000 r.p.m. (1,400 × g) for 15 minutes in a PR-2 International centrifuge at approximately 0° C.

Method IVB: Following preparation of the material as in *method IVA*, the supernate was centrifuged again at 9,500 r.p.m. (7,000 × g) for 15 minutes.

Method IVC: The material to be inoculated was prepared as in *method IVB* and was passed through a Selas #03 filter.

The materials so prepared were injected immediately, unless otherwise indicated in the tables. Injections were done subcutaneously. A 26-gauge, 1½-inch needle was introduced in the right hind region through the upper muscles of the hind leg so that leakage was minimized; 0.1 cc. of the test material was deposited in the region of the nape of the neck.

Newborn litters, less than 24 hours old, obtained from pedigreed matings, were used as test mice. Where possible, alternating litters were used as control mice; *e.g.*, all members of the first litter were used as experimental mice, the second litter serving as controls, or *vice versa*. The various prepared materials were injected simultaneously into 4-week-old mice of the strain from which leukemic tissues were obtained: AKR, C58, or C3H/Fg. This was done to test for the presence of viable cells in the preparations. The mice were killed when ill, or near death from leukemia, parotid neoplasms, or other tumors, and were autopsied at once to avoid post-mortem changes. All the organs of the autopsied mice were carefully examined. The parotid gland and the exorbital lachrymal gland were taken routinely for microscopic study. Other representative sections were taken on numerous groups of mice, and in all mice, lesions observed on gross examination were sectioned for microscopic study. Specimens were fixed in Tellyesniczky's fluid (70 percent ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part) or they were fixed in formalin-Zenker's fluid. The sections were stained by hematoxylin and eosin, and special stains were used for selected material, as described in the section on morphology.

Results

The results will be given separately for Groups I, II, and III. Group I comprises strain $C3H_f$ mice; Group II, $(C3H_f \times AKR)F_1$ hybrids; and Group III, $(C3H_f \times C3H/Fg)F_1$ hybrid mice.

Group I

The response of strain $C3H_f$ mice to the various preparations of leukemic tissues is shown in table 1, and a record of the individual experiments in which leukemic mice or mice having parotid neoplasms were observed is given in table 2.

TABLE 1.—*Leukemia and pleomorphic tumors of the parotid gland in $C3H_f$ mice given extracts, centrifugates or filtrates of leukemic tissues from high leukemic strains*

	Number of mice	Number of separate litters	Leukemia			Parotid tumors		
			Number	Percent	Age at death (*months*)	Number	Percent	Age at appearance (*months*)
Experimental..	283	55	14*	4. 9	17. 3	22	7. 7	11. 7†
Control.....	161	32	6‡	3. 7	20. 8	None		

*Includes 9 lymphocytic neoplasms, 3 Hodgkin's-like lesions, 1 reticulum-cell sarcoma, and 1 plasma-cell leukemia.

† Parotid-gland tumors appeared in only 8 of 55 litters. See text and table 2.

‡ Includes 5 lymphocytic neoplasms and 1 Hodgkin's-like lesion.

There was no increase in the incidence of leukemia in the experimental group over the controls. Leukemia occurred as a rule in older mice and was not confined to any particular litters. An exception was found in 3 mice having leukemia at 3, 7, and 7 months, respectively, which preceded any leukemic deaths among the control group. Although female #66 ($C3H_f$, experiment 79), leukemic at 3 months, presented all the clinical and morphologic features of a typical acute lymphocytic neoplasm, including an extremely high level of leukemic cells in the peripheral blood, transplants of leukemic tissues did not grow progressively in AKR or $C3H_f$ mice. Eight other leukemias in this experimental group grew progressively following transplantation in mice of the $C3H_f$ strain but not in mice of the donor strain of leukemic tissue, AKR or C58.[4]

Of particular interest was the occurrence among $C3H_f$ mice given extracts, centrifugates or filtrates of leukemic tissues, of 22 (7.7 percent) pleomorphic tumors of the parotid gland. These neoplasms were usually bilateral, in time reaching massive size, and occurred as early as 4 months and as late as 19 months of age. The parotid tumors were found in only 8 of the 55 experimental litters. All the mice of 5 different litters had

[4] The genetic laws of transplantation relating to neoplastic or normal tissue are well established (*13, 14*). Neoplastic tissue will grow progressively only within the inbred strain (or specific subline) in which it arose and in F_1 mice obtained from crosses employing this particular inbred strain as one parent. A certain number of F_2 hybrid and backcross mice will grow the neoplasm progressively, depending upon the number of histocompatibility genes concerned. Exceptions are known and the mechanisms determining these exceptions have been elucidated. The AKR and C3H strains share the so-called "strong" histocompatibility gene $H\text{-}2^k$ (*15*); thus, the probability of progressive growth in transplantations between these 2 strains is greater than transplantations between strains (*e.g.*, C58 and C3H) which have different alleles at this H-2 locus. Nevertheless, this is a method which can be used to distinguish between growths arising *de novo* in the inoculated host (spontaneous or induced neoplasms) on the one hand or neoplasms growing as a result of the transfer of viable cells (*16, 17*).

TABLE 2.—*Experiments in which leukemia or parotid-gland neoplasms were found in C3H$_f$ test mice*

Experiment	No. of mice	Age of mice inoculated	Source of material	Method of preparation	Neoplasms	
					Parotid gland (sex and age at appearance)	Leukemia (sex and age at death)
8	5	< 12 hrs.	AKR—T^{22}*	I†	♂–9 mo. ♀–9 mo. ♂–18 mo. ♂–12 mo. ♂–18 mo.	
13	9	<12 hrs.	C58—T^{20}	II	———	♂–24 mo.
14	7	<12 hrs.	AKR—T^{8}	II	———	♀–22 mo.
20	7	<12 hrs.	C58—T^{14}	IIIA	———	♂–24 mo.
23	5	<6 hrs.	C58—T^{15}	IIIA	———	♀–26 mo.
24	5	<24 hrs.	AKR—T^{10}	IIIA	———	♂–24 mo.
25	8	<24 hrs.	AKR—T^{37}	IIIA	———	♀–23 mo. ♀–25 mo.
26	8	<24 hrs.	C58—T^{25}	IIIA	———	♂–13 mo.
54	3	<12 hrs.	AKR—T^{33}	II	♂–14 mo. ♀–16 mo. ♀–18 mo.	
57	5	<20 hrs.	AKR—T^{35}‡	II	♀–5 mo.§ ♀–5 mo. ♂–6 mo. ♀–6 mo. ♀–7 mo.	
58	2	<6 hrs.	AKR—T^{35}‡	II	♀–12 mo. ♀–12 mo.	
59	1	<12 hrs.	C58—T^{40}	II	———	♂–10 mo.
63	4	<12 hrs.	C58—S‖	IVB	♀–16 mo. ♀–16 mo.	
64	2	<12 hrs.	C58—S‖	IVB	♀–4 mo. ♂–8 mo.	
67	2	<18 hrs.	AKR—S	IVA	———	♀–16 mo.
68	8	<12 hrs.	AKR—S	IVA	♀–19 mo.	
71	7	<4 hrs.	AKR—S	IVC	♂–7 mo.¶ ♀–13 mo.§	♂–7 mo.
79	6	<24 hrs.	AKR—S	IVC	———	♀–3 mo. ♂–7 mo. ♀–18 mo.

*Leukemic tissues obtained either from the high-leukemic AKR or C58 strains; T=transplantable leukemia. Superscript refers to transfer generation. S=spontaneous leukemia.

† See Materials and Methods for a description of preparations of materials used to inoculate C3H$_f$ test mice.

‡ Same centrifugate used for litters in experiments 57 and 58. C3H$_f$ test mice obtained from different pedigreed mothers.

§ ♀ 23 had an adrenal medullary neoplasm in addition to bilateral parotid tumors at 13 months of age; ♀ 1 had an adrenal medullary neoplasm in addition to parotid tumors at 5 months of age.

‖ Same centrifugate used for litters in experiments 63 and 64. C3H$_f$ test mice obtained from different pedigreed mothers.

¶ ♂ 20 had a lymphocytic neoplasm and bilateral parotid tumors at 7 months of age.

parotid-gland tumors. (See experiments 8, 54, 57, 58, and 64 in table 2.) No sex differences were noted among this group.

These pleomorphic tumors have not been observed in the 2 sublines of the C3H strain employed in this study, nor in the high-leukemic C58 and AKR strains. Two such neoplasms of the usual morphologic structure have been observed among 50 mice in the C3H/Fg subline in our laboratory.

Two mice in the experimental group with bilateral tumors of the parotid gland also had anaplastic carcinomas of the adrenal medulla. These occurred in females, 5 months [5] and 13 months of age, in different experiments (experiments 57 and 71). $C3H_f$ ♂ #20 had bilateral parotid tumors and lymphocytic leukemia at 7 months of age (experiment 71).

There appears to be no relationship between pedigreed litter of $C3H_f$ test mice, source of leukemic material (whether C58 or AKR strain, spontaneous or transplantable) or method of preparation of leukemic material, and the induction of parotid-gland neoplasms. Though in several instances (see table 2), entire litters developed parotid-gland tumors, prior or subsequent litters from the same pedigreed mothers did not develop these neoplasms. In experiment 8 extracts of AKR-leukemic tissues from the transplantable line L4616 induced parotid neoplasms, yet in 3 other experiments with this transplantable line the test mice were negative. In experiments 57 and 58 centrifugates from leukemic tissue of another AKR transplantable line induced parotid neoplasms in all 7 mice, comprising 2 litters obtained from different pedigreed mothers, yet on 4 other occasions such preparations failed to elicit these tumors.

There were 11 histologically diagnosed AKR strain spontaneous lymphocytic leukemias and 7 C58 strain spontaneous leukemias used in 26 separate experiments; in 29 experiments 3 different lines of AKR transplantable leukemias and 3 different C58 lines of various transfer generations were used as sources of leukemic materials.

Thirty-three F_1 and F_2 offspring have been obtained from 2 females (♀6, experiment 8, and ♀4, experiment 57) that had bilateral parotid-gland tumors at the time of delivery and during the period of nursing. The youngest of these descendants is now 17 months old and none has developed tumors of the parotid gland.

Fifteen of these pleomorphic parotid-gland tumors in $C3H_f$ mice have been transplanted to subcutaneous sites. To date, 8 have grown progressively, at least in the first transfer, in $C3H_f$ mice 4 to 6 weeks of age, but not in mice of the AKR or C58 strain—the donor strains of leukemic tissues (see table 3). The morphologic description of those parotid tumors grown successfully following transplantation is given under the section on morphology. Two of these have been transferred through the third and fifth transfers (tumors VIII and XII) and regularly kill the host $C3H_f$ mice in approximately 30 days.

[5] One of the 2 adrenal medullary carcinomas grew progressively upon transplantation in $C3H_f$/Lw but not in AKR mice. (Tumor L5665 which appeared in $C3H_f$/Lw ♀1 at 5 months of age). This neoplasm has now been carried serially in transplantation through 23 passages, and filtrates of tumorous tissue grown subcutaneously in $C3H_f$ mice have proved to be a good source of parotid-gland tumor-inducing substance.

TABLE 3.—*Results of transplantation of pleomorphic tumors of the parotid gland of strain C3H$_f$*

Expt.	Tumor designation	Sex and age of mouse with tumor (*months*)		$C3H_f$ mice			AKR mice		Remarks
				No. inoculated	No. positive	Latent period	No. inoculated	No. positive	
		Sex	*Age*						
	I	♂	18	3	0	—	3	0	No progressive growth in 27 months.
	II	♂	9	3	1	5 mo.	3	0	No growth in 2nd transfer.
8	III	♂	12	3	1	17 mo.	3	0	Now in 4th transfer.
	IV	♀	9	3	0	—	—	—	No progressive growth in 27 months.
	V	♂	18	3	0	—	3	0	
54	VI	♀	16	2	2	4 mo. 7 mo.	3	0	Now in 4th transfer.
	VII	♀	6*	3	0	—	3	0	
	VIII	♀	5	3	2	10 mo. 10 mo.	3	0	Now growing in 6th transfer; kills at 1 month.
57	IX	♀	5	7†	0	—			
	X	♂	6	5	1	5 mo.			Not carried in transfer.
	XI	♀	7	3	0	—			
58	XII	♀	12	3	2	6 mo. 10 mo.			Growing progressively in 3rd transfer.
	XIII	♀	12	3	0	—	2	0	
	XIV	♀	4	3	1	7 mo.	3‡	0	
64	XV	♂	8	3	0	—	3	0	No growth in 2nd transfer.

*An adrenal medullary neoplasm present in this animal grew progressively in $C3H_f$ mice and has been maintained in transfer for 23 generations. See footnote 4, p. 498.
†Parotid neoplasms transplanted from both right and left sides.
‡Strain C58 mice used here since source of leukemic tissues was the C58 strain.

Other Tumors

Some $C3H_f$ mice are still alive at an average age of 28.5 months in the experimental and control groups. Other neoplasms common to $C3H_f$ mice have been observed in both groups: 16.9 percent mammary tumors in 150 females of the experimental group at an average age of 18.8 months and 13.8 percent mammary tumors in 101 control females at an average age of 20.3 months. Males in both groups had a significantly higher incidence of hepatomas than females. The incidence for both sexes among the experimental series was 37 percent. The average age at autopsy was 21.5 months. There were observed 35 percent hepatomas in the control group at 21.0 months. In addition, ovarian granulosa-cell tumors and adrenal cortical adenomas were the next most common forms found approximately in equal numbers in both groups.

Mortality among the $C3H_f$ mice inoculated when less than 24 hours old with various preparations of leukemic materials has not been excessive. Inoculations of homogenized extracts (*method I*) produced 59 percent deaths but these were mostly leukemic deaths. In 43 litters given centrifugates or filtrates, only 15.3 percent of the young mice died before maturity; none died from leukemia. The numbers of mice listed in the tables refer to those surviving at the time of the first appearance of leukemia or parotid tumors.

Group II.—($C3H_f$ × AKR)F_1 Mice

Females of subline $C3H_f$/Lw were mated with AKR males to obtain the ($C3H_f$ × AKR)F_1 mice discussed in this group. These F_1 mice, as observed in this laboratory, develop spontaneous leukemia, mostly lymphocytic, beginning at 7 months of age. In a sample of 165 F_1 mice (83♀♀ and 82♂♂) there was observed an incidence of 56.4 percent. Leukemic deaths occurred at an average age of 14.3 months. No striking sex differences in incidence or age at death was observed. A small number of mammary tumors and hepatomas have been observed among the nonleukemic mice.

There are 113 F_1 hybrid mice (61♀♀ and 52♂♂) now 15 to 18 months of age in this group, comprising 17 experimental litters. These were inoculated when less than 24 hours of age with extracts, centrifugates or filtrates of leukemic tissues from the following strains of mice: AKR, C58, C3H/Fg and a separate subline of the AK strain, AK-n.[6]

Five spontaneous leukemias from the AKR strain and 2 transplanted leukemias of this strain, 1 spontaneous C58 leukemia, 3 spontaneous leukemias of the C3H/Fg subline, 4 spontaneous leukemias of the AK-n strain, and 2 transplantable leukemias of this strain were used as sources of leukemic tissues in the 17 separate experiments.

All leukemias were diagnosed histologically as lymphocytic neoplasms. One leukemia of spontaneous origin, obtained from the C3H/Fg subline, and used in experiment GH–22, ($C3H_f$ × AKR)F_1 mice, and also in experiment GH–21, ($C3H_f$ × C3H/Fg)F_1 mice (see group III, next section), had features resembling reticulum-cell neoplasm, type B, or the Hodgkin's-like lesion described by Dunn (*9*). Another spontaneous leukemia, obtained also from the C3H/Fg subline, and used in experiment GH-26, had similar morphologic features.

To date, 21 leukemias (18.6 percent) and 9 pleomorphic parotid-gland tumors (7.9 percent) have been observed. The average age at death of the leukemic mice was 10.2 months and the average age at appearance of parotid-gland tumors, 7.9 months. Table 4 lists the experimental litters in which either leukemia or parotid-gland tumors were found. Two mice had both leukemia and bilateral parotid tumors; a male had bilateral parotid tumors and a subcutaneous growth; and a female, 3

[6] AK-n mice obtained through the courtesy of Dr. Ludwig Gross.

TABLE 4.—*Leukemia and pleomorphic tumors of the parotid gland in F_1 mice ($C3H_f \times AKR$) given extracts and filtrates of leukemic tissues*

Expt.	No. of mice	Age inoculated (*hours*)	Source of leukemic material	Method of preparation	Parotid neoplasms (sex and month appeared)	Leukemia (sex and month at death)
GH–1	6	<12	AKR—S*	I†		♂–11 mo. ♀–11 mo. ♂–17 mo.
GH–3	7	<20	AKR—S	I		♀–6 mo. ♂–11 mo. ♂–8 mo. ♂–14 mo. ♀–10 mo. ♂–18 mo. ♀–10 mo.
GH–6	10	<12	AKR—T^3	I	♀–3 mo.‡ ♀–5 mo.§ ♀–8 mo. ♀–11 mo.	♀–5 mo.§ ♀–6 mo. ♀–8 mo.
GH–9	7	<6	AK-n—S	IVA		♂–7 mo. ♂–11 mo. ♂–9 mo. ♀–13 mo.
GH–14	8	<20	C3H/Fg—S	IVB		♀–11 mo.
GH–17	8	<6	AKR—T^4	IVB‖	♂–11 mo.§	♂–11 mo.§ ♂–13 mo.
GH–18	5	<2	AK-n—T^5	IVA‖	♂–4 mo.	
GH–22	5	<24	C3H/Fg—S	IVB	♂–4 mo. ♂–12 mo.‡ ♀–9 mo.	
GH–24	8	<4	C3H/Fg—S	IVB‖		♂–4 mo.

*Leukemic tissues obtained from various leukemic strains; S=spontaneous; T=transplantable. Number refers to transfer generation used.

†See text for description of method of preparation of tissues.

‡Mouse ♀ 30, experiment GH-6, with bilateral parotid neoplasms at 3 months of age also had a mammary adenocarcinoma. ♂ 139, experiment GH-22, which had bilateral parotid tumors also had a fibrosarcomatous growth in close proximity to the right parotid tumor. See section on morphology of tumors.

§These 2 mice had bilateral parotid tumors and leukemia at autopsy.

‖Mice in these experiments given 400 r X ray prior to inoculations.

Table includes only those litters in which lymphocytic neoplasms or tumors of the parotid gland were found.

months of age, had a mammary adenocarcinoma in addition to bilateral parotid tumors.

It is of interest to note that there were 5 (20 percent) leukemic deaths of (C3H$_t$ × AKR)F$_1$ mice of the experimental group before any leukemic deaths among 50 uninoculated control mice. These occurred between the 4th and 7th month as follows: 1 at 4, 1 at 5, 2 at 6, and 1 at 7 months. At the 18th month, however, the control group had an incidence of leukemia of 54 percent and an average age at leukemic death of 13.7 months, while the incidence of leukemia among the 113 experimental mice was only 18.6 percent.

All leukemias in this group were found to be of the lymphocytic form. Five of these, all appearing in mice less than 8 months of age, were transplanted to (C3H$_t$ × AKR)F$_1$ mice and to AKR mice (source of leukemic-tissue preparation inoculated). Four grew progressively only in the F$_1$ mice, killing in from 25 to 50 days. One, which appeared at 5 months in ♀31, experiment GH-6, did not grow in F$_1$ or AKR mice.

Seven parotid-gland neoplasms were transplanted to (C3H$_t$ × AKR)F$_1$ mice and to mice of the donor strain of leukemic tissue. Progressive growth has been obtained in F$_1$ mice in only two of these to date; however, the elapsed time since transplantation is now only 7 to 9 months.

Results of Inoculations of Filtrates Prepared from Lymphocytic and Parotid-Gland Neoplasms Arising in Group I (C3H$_t$) and Group II (C3H$_t$ × AKR)F$_1$ Mice

Filtrates from 5 different lymphocytic neoplasms [3 C3H$_t$ mice and 2 (C3H$_t$ × AKR)F$_1$ hybrids], prepared as described under *method IVC*, were inoculated into C3H$_t$/Lw mice of 5 separate litters. Recipient mice ranged from 6 to 24 hours of age at inoculation. No lymphocytic neoplasms or parotid tumors have appeared in this group of mice now 12 to 15 months of age. In addition, filtrates from 4 different bilateral parotid tumors of C3H$_t$/Lw mice, prepared in the same manner were similarly inoculated into C3H$_t$ and (C3H$_t$ × AKR)F$_1$ mice. Only one parotid tumor has been observed, and it appeared at 12 months of age in a (C3H$_t$ × AKR)F$_1$ test mouse. These results are strikingly different from those obtained using filtrates of a transplantable adrenal medullary neoplasm (see footnote 5, p. 500).

Group III.—(C3H$_t$ × C3H/Fg)F$_1$ Mice

These F$_1$ mice were obtained by matings of ♀C3H$_t$/Lw × ♂C3H/Fg. The reciprocal cross was not studied since the Fg subline of C3H mice carries the mammary tumor milk-agent and the presence of mammary tumors would complicate a study of the induction of neoplasms. We have observed to date nearly 30 percent leukemia in C3H/Fg mice of two morphologic forms: lymphocytic and a reticulum-cell neoplasm variant, the Hodgkin's-like lesion.

Twenty-eight F$_1$ mice in six separate litters were given inoculations of leukemic preparations when less than 24 hours old. Uninoculated control

mice were obtained from the same matings and are either prior or subsequent litters. All were obtained from six pedigreed $C3H_f$/Lw mothers: Females #15607, #15608, and #15612 were litter mates and females #15250, #15253, and #15254 were litter mates. Three different C3H/Fg fathers were used and these also were litter mates. Survivors of the present group of F_1 mice are now 11 to 15 months of age. Tumors have been observed to date in only 3 experimental litters and the results are shown in table 5. Fifty percent of the litters and 36 percent of the mice have developed parotid-gland neoplasms. All but two were bilateral and recognized grossly. These appeared as early as 5 and as late as 12 months. In addition, 5 F_1 mice of experiment GH-21, 2 in GH-26, and 2 in experiment GH-32 (a total of 9, 4 females and 5 males) have presented sub-

TABLE 5.—*Neoplasms in F_1 mice ($C3H_f \times C3H/Fg$) given extracts and filtrates of leukemic tissues**

Expt.	No. of mice	Age inoculated (*hours*)	Method of preparation	Source of leukemic material	Parotid tumors (sex and age at appearance)	Subcutaneous neoplasms
GH-21	6	24	IVB	C3H/Fg—S†	♀–8 mo. ♀–8 mo. ♂–10 mo. ♀–11 mo. ♀–12 mo.	All mice with parotid tumors had subcutaneous growths.‡
GH-26	2	6	IVA	C3H/Fg—S	♂–5 mo.§	♂3, ♂5 had subcutaneous growths.
GH-32	7	12	IVA	C58—S	♂–8 mo. ♀–6 mo. ♂–9 mo. ♂–10 mo.	♂9, ♂10 had subcutaneous growths.

*Table includes only those litters in which tumors of the parotid gland or subcutaneous sarcomas were found.
†Preparation stored for 30 hours at 4° C. Same material used immediately in experiment GH–22 (table 4).
‡All subcutaneous tumors found in this group of ($C3H_f \times C3H/Fg$) F_1 mice were fibrosarcomatous except ♂ 167, experiment GH–26, which was autopsied at 3 months of age and whose parotid glands appeared normal. Two growths, one in the right axilla and one at the right inguinal region, resembled the pleomorphic growths found in the parotid gland itself. See discussion under morphology of tumors.
§Early neoplasm of parotid gland—microscopic.

cutaneous growths. One animal with a subcutaneous growth had a microscopic parotid neoplasm; in another the parotid glands were normal histologically (experiment GH-26). The subcutaneous growths were found dorsally and were located, in most instances, near the inguinal region. Two were located near the axillary region. One subcutaneous growth, for example, in female #134 (experiment GH-21), 12 months of age, was found in the left flank in the region of the 3rd and 4th mammary glands, extending posteriorly. It measured 10 × 40 mm. and was 14 mm. thick. A second subcutaneous growth was found in the region of the fifth mammary gland on the right flank. This growth measured 10 × 20 mm. and was 10 mm. thick. This mouse also had bilateral parotid-gland tumors which were 20 mm. in diameter.

All except one of these subcutaneous neoplasms were sarcomatous with unusual infiltrative capacity. Subcutaneous growths in male #167 (experiment 26) which appeared at 3 months of age reproduced the characteristic morphology of the parotid-gland tumors. The parotid glands in this animal, however, were normal histologically. The morphologic features of these subcutaneous tumors, as well as the one observed in $(C3H_f \times AKR)F_1$ mice, are discussed in the section on morphology.

Four out of 5 subcutaneous growths grew progressively upon transplantation into $(C3H_f \times C3H/Fg)F_1$ mice, but failed to grow in C3H/Fg mice (experiments GH-21 and 26) or in C58 mice (experiment 32), the donor strains of leukemic tissue. Two tumors are now in the third transfer, and one in the fourth, and they kill in approximately 30 days, with massive subcutaneous growths.

None of four parotid-gland tumors, in this series, has grown progressively after 4 months from date of transfer.

One lymphocytic neoplasm in the experimental series of 28 mice and 1 in 20 controls have been observed. No other neoplasms have appeared to date in this group of F_1 mice.

An additional 46 litters of experimental mice, comprising groups I, II, and III of test animals, are under observation. These are not included in the tabular material or discussion because the average age of the mice is now only 12 months. In order to give some idea of the number, distribution, and age of mice at appearance of these pleomorphic tumors of the parotid gland in our total experience to date, the data, including 46 litters still under observation, are given in table 6. It may be seen that 1 litter in 5 contained at least 1 mouse with a parotid-gland tumor. There were 56 tumors distributed among 23 litters. There were 132 mice in the positive litters so that 42.4 percent of the mice in positive litters had tumors of the parotid gland.

TABLE 6.—*Number, distribution, and age of mice with pleomorphic parotid-gland tumors among several experimental groups in total experience to date*

Number of litters under observation	Total number of mice	Parotid tumors		Litters with parotid tumors		Number mice in litters positive for tumors	Age of mice with tumors (*months*)	
		Number	Percent	Number	Percent		Average	Range
114	692	56	8.1	23	20.1	132	9.8	3–19

Morphology of Tumors of the Parotid Salivary Gland

Previous Reports of Tumors of the Salivary Glands in Mice

a) Spontaneous myoepitheliomas.—Tumors designated "myoepitheliomas" have been observed repeatedly in this laboratory in strains A and BALB/c, and in no other strain except for two cases in strain C58. We have no exact incidence figures for large groups of mice. Three myoepi-

theliomas were found in 86 female strain BALB/c, and four in 128 BALB/c × C3H hybrids examined for mammary tumors (*18*). Fekete and Kirschbaum reported lines of transplanted salivary-gland tumors in strain BALB/c (*19*). No record of a myoepithelioma or any other tumor of the salivary glands has previously been found among several sublines of strain C3H mice at the National Cancer Institute, although a recent survey was made in which all subcutaneous tumors were examined microscopically from 736 strain $C3H_t$/He females of an average age of 18.6 months (*20*).

The tumors called myoepitheliomas previously observed in this laboratory have usually grown too extensively at the time of autopsy for the exact site of origin to be determined, but microscopic sections of smaller tumors have shown parotid-gland tissue at the margin, which makes it probable that the parotid was the gland in which they originated. The morphology of these tumors is distinctive (fig. 1). Characteristically the tumors formed large, central cysts, containing a glairy, mucoid substance which adhered to the knife. The histologic pattern showed a rough stratification of cells so that a basal, rounded cell lay adjacent to the connective tissue stroma and vessels. The cells above were progressively more flattened, and fusiform (fig. 2). Microscopic sections of the entire tumor have a similar histologic pattern in all areas. Special stains have been made of the tumor cells, and myofibrils were revealed (*21*). These myoepitheliomas in the mouse resembled areas in tumors of the parotid gland in man described by Sheldon (*22*). The tumors in the mouse have been repeatedly transplanted and maintained for a number of generations. The transplants have usually developed to about ½ cm. in size in 3 weeks and the host may survive 6 to 8 weeks. The transplanted tumors closely resembled the original tumors in histologic structure and formed central cysts filled with a slimy, mucoid substance similar to that observed in the primary tumors. A remarkably constant effect of these tumors, both primary and transplanted, was a granulocytic leukemoid reaction in the host (*23*).

The two myoepitheliomas observed in strain C58 have differed in that they have many small cysts throughout the tumor, rather than a large, single, central cyst. One has been transplanted successfully and has not shown a leukemoid reaction.

b) Tumors of the salivary glands induced by carcinogens.—Bauer and Byrne (*24*) reviewed preceding reports on the induction of tumors of the salivary glands, and described the results of their own experiments. Methylcholanthrene; 9,10-dimethyl-1,2-benzanthracene; 1,2-benzanthracene; and 1,2,5,6-dibenzanthracene, in pellets of beeswax were implanted in the salivary glands of rabbits, and of mice belonging to strains C57BL, A, BALB/c, and AK. No malignant tumors developed in the rabbits, but "proliferation of ductal epithelium and new duct formation in dense, hyalinized, cartilage-like tissue" was observed. In mice "the hyalinization of the tissue adjacent to the pellet was not so pronounced as in some rabbits." Numerous tumors developed in mice. Most of these were

adenoacanthomas. The effort to incite the so-called "mixed tumor" (pleomorphic adenoma) was unsuccessful. Even in small tumors in mice, proliferation of ductal epithelium was noticeable, while participation of acinous cells was uncommon. The site of origin of the initial metaplastic change and of the subsequent neoplasm was considered to be the "pluripotential cuboidal epithelial cell of the intercalated ducts."

c) *Pleomorphic or "mixed" tumors of the salivary gland.*—The commonest group of tumors of the salivary gland in man has long been referred to as "mixed." Originally this implied a derivation of the tumor from two or more types of tissue. In a recent review Foote and Frazell (*25*) stated that they used the term in "a descriptive sense only." Willis (*26*) considered the term a misnomer, and preferred the purely descriptive adjective "pleomorphic," since it had no "erroneous connotation." A similar difficulty arises in supplying a name for the tumors found in the treated mice in this experiment. Pleomorphic is appropriate if it is understood to mean a tumor occurring in different forms, and having various morphologic components. No previous report has been found of similar tumors of the salivary glands in mice, or in any other laboratory animal except the dog. In this species "mixed" tumors of the salivary gland resembling those in man were referred to briefly by Harvey *et al.* (*27*). No tumors of this type were found in control mice in the present experiment, while spontaneous tumors morphologically similar to those to be described have been found only twice, as mentioned previously, in untreated mice of the subline C3H/Fg, and once in a (C3H $\times$ C58)F_1 female 1 year of age. Woolley reported similar tumors in mice treated with cortisone (*28*).

Morphology of the pleomorphic tumors of the parotid salivary gland found in treated mice.—Foote and Frazell (*25*) in their review of tumors of the salivary glands in man considered it "preferable to illustrate generously many of the microscopic features" rather than to repeat elaborate descriptions. The same procedure is desirable for these pleomorphic parotid-gland tumors in the mouse. The great variety of patterns makes it difficult to describe them in words and photographs are relied upon. Although different areas in individual tumors showed great diversity, the tumors as a group were remarkably similar and they are easily identified by a number of characteristic features. They will be described as a group, with special notation of a few exceptional tumors. No difference was detected in those from the strain $C3H_f$ mice, the F_1 hybrid groups, the two spontaneous cases in the C3H/Fg subline, or the one in the C3H $\times$ C58 hybrid.

Macroscopic.—The tumors were for the most part bilateral and usually multinodular, often resembling a bunch of grapes (fig. 3). Throughout the smaller tumors and in areas of larger tumors the nodules were usually of similar size, often confluent and with indistinct outlines. In larger tumors one or several nodules appeared to outgrow the others, and some nodules attained sizes of a centimeter or more, so that the multinodular character evident in the smaller growths was obscured. Usually the

tumors were easy to separate from the surrounding tissue, except for a few especially noted later, which were adherent to the overlying skin. The color of the larger nodules was usually grayish-white. Smaller nodules were sometimes pearly-white. Hemorrhagic discoloration was infrequent. In consistency the nodules varied. The smaller ones were sometimes firm, but larger nodules were usually rather soft and exuded a watery fluid when sectioned. Cysts filled with clear, opalescent, or brown mucoid fluid could sometimes be seen within the larger nodules. The tumors usually extended laterally, forming an enlargement in the neck below the ear. The sublingual gland has not been found involved in the neoplastic change; the submaxillary has been involved once; while the exorbital lachrymal gland has frequently shown a neoplastic change, similar to and concomitant with the change in the parotid salivary gland.

Microscopic.—On microscopic examination, a great variety of histologic patterns has been found in individual tumors, yet certain patterns tend to be repeated regularly in the group as a whole. Both an epithelial and a mesenchymal or fibroblastic component could usually be distinguished. The epithelial component consisted generally of small tubules or minute cysts lined by a cuboidal epithelium. The other component showed great diversity, ranging from a poorly differentiated mesenchymal type of tissue resembling fetal connective tissue, with basophilic fusiform cells separated by a loose network of fibers, to a well-differentiated compact tissue composed of elongated fibroblastic cells with eosinophilic cytoplasm and intercellular collagen. Some of the most frequent patterns of the tumors are photographed and described as follows:

Sections of the entire tumor.—The multinodular nature of the growth was apparent (fig. 4). A cervical lymph node, included in the section, had not been invaded by tumor tissue. This was characteristic, since the tumors showed little tendency to invasive growth. Normal parotid tissue remained in the center, and a tongue of normal tissue surrounded some of the neoplastic nodules. Cyst formation, and apparent encapsulation of the nodules were seen. In one area there was a massive accumulation of a characteristic hyaline substance. Another cross section of a larger tumor (fig. 5) also showed the multinodular and multicentric nature of the growth. Some normal gland tissue remained. A series of photographs at a high magnification was taken from separate areas of this tumor, in order to show cellular details and to illustrate the pleomorphic structure. The first (fig. 6) showed what was probably the most distinctive pattern in this group of tumors. Small indistinct tubules were formed by cuboidal epithelium. The epithelial tubules were surrounded by and separated from each other by a mesenchymal type of tissue composed of fusiform and oval cells. Mitotic figures were usually abundant in the mesenchymal tissue. The next area (fig. 7) showed an amorphous hyaline material, which was especially frequent in smaller tumors, and in central areas of large tumors, but generally absent from the largest nodules. This material stained heavily with eosin; it was positive by the periodic acid-Schiff technique, both with and without diastase; it

showed no metachromasia with toluidine blue except in small areas of very heavy deposits, and it was colored an intense green by the fast green dye in Masson's trichrome stain. Cysts were an important feature in these tumors and the margin of one of the cysts in cross section is shown (fig. 8). A mesenchymal type of tissue surrounded it. Cells lining the cysts were often necrotic, but where preserved, as in the present illustration, they were usually flattened. In some small cysts the lining cells were cuboidal. The present tumor contained areas formed of elongated fusiform cells with collagenous intercellular substance (figs. 5 and 9). The component cells appeared to be more differentiated and resembled mature fibroblasts more closely than was usual in other tumors of this type. Areas like the one pictured resembled fibrosarcomas commonly found in the mouse. Reticulum fibers lying between and closely investing the fusiform cells could be demonstrated by Wilder's method. Another section from this tumor showed considerable infiltration with normal-appearing lymphocytes (fig. 10). The lymphocytes might be clumped together, as in the area shown here, or diffusely scattered throughout a nodule. If a single section only was examined, the lymphocytic infiltration was sometimes sufficiently extreme to resemble a lymphocytic neoplasm.

Characteristic patterns from other tumors of this pleomorphic type are shown in ensuing photographs. In figure 11, areas of heavily packed cells at the periphery of a frond-like or lobular formation were sharply demarcated from areas in the interior in which nuclei were sparse and widely separated. Similar patterns with a central light area and denser peripheral area were frequent in larger tumors and in some transplanted tumors. Figure 12 is from another primary tumor. A variation in cell density, with more concentration of cells about blood vessels formed a distinctive pattern. A similar pattern was especially frequent in transplanted tumors. Sometimes this pattern resembled the myoepithliomas previously described in having a rounded cell adjacent to the vascular stroma, and a flattened, more eosinophilic cell at a distance from the vessels. Figure 13 shows a frequently observed pattern in the large nodules. Delicate strands of cells were widely separated by vacuoles which often contained material which was blue after hematoxylin and eosin staining, but was not metachromatic with toluidine blue. A transition between an area with many tubules to an area of solidly packed oval cells without a glandular pattern is shown in figure 14. Figure 15 is taken from the skin overlying the parotid gland, which contained a tumor adherent to the skin. A fibrosarcomatous type of tissue, resembling that shown in figure 9, developed from, and in close association with, the parotid-gland tumor and infiltrated the skin.

Fat stains (Sudan IV after formalin fixation) were done on a representative group of tumors. None of the viable tumor areas were positive. Necrotic tissue was sometimes stained by Sudan and heavy deposits of hyaline substance were faintly tinged.

Features of these tumors, which are important in the attempt to trace

histogenesis are shown in figures 16 to 23. Figure 16 illustrates the smallest tumor found. It was not noted on gross examination. In one lobule of the parotid gland all acini had disappeared and only small tubules lined by cuboidal epithelium were found. Between the tubules were fusiform cells, plasma cells, and the characteristic amorphous hyaline substance. Two nodular foci in this tumor were composed predominantly of fusiform cells in the center with only a few tubules present at the periphery. Another nodule showed a considerable amount of the amorphous hyaline substance lying between tubules. Several small cysts were developing, apparently as dilatations within the lumen of tubules. In the largest of these the epithelium was flattened, and a considerable amount of hyaline material surrounded it. Overlying muscle was pushed outward but not infiltrated by the expanding tumor. Figure 17 showed the disappearance of acini in the exorbital lachrymal gland, and replacement by tubular structures. A periodic acid-Schiff preparation of the same area showed the tubules filled with Schiff-positive substance (fig. 18). A similar fading out of acini could often be observed in the parotid gland, and it was once observed in the submaxillary gland (fig. 19), but it showed up more strikingly in the exorbital lachrymal gland where the acini are larger. The exorbital lachrymal glands in old untreated mice of the control group sometimes showed small areas of atrophy, in which the acinar cells had disappeared, but the process was much more pronounced when a neoplastic change was present. The disappearance of acini in association with the neoplastic changes after introduction of carcinogens in the salivary glands was noted by Bauer and Byrne (*24*). In the present material an infrequent incidental finding in the exorbital lachrymal gland of both treated and control mice was the inclusion of small areas of Harderian-gland tissue.

A nodule in which hyaline substance was abundant and rather evenly distributed is shown in figure 20. In the nodule in figure 21 the substance was apparently being pushed to the periphery by the expanding growth of fusiform cells in the center. In figure 22 margins of three nodules were shown with a small rim of hyaline substance. Wilder's method for reticulum (fig. 23) showed an accumulation of fibers at the margin of nodules. This series suggested that the hyaline substance was associated with a preneoplastic or early neoplastic change, an opinion which was supported by the work of Bauer and Byrne on the induction of salivary gland tumors with carcinogens. Within the hyaline nodule, a focus of more rapidly growing tissue composed of epithelial tubules and fusiform cells appeared to develop. This secondary focus, growing expansively, pushed the hyaline substance to the periphery, where it formed a pseudo-capsule. Smaller nodules of tumor tissue were surrounded by a capsule probably formed by compressed tissue.

Small discrete nodules of tumor were sometimes found in adjacent mammary-gland tissue and fat. It is problematic whether this represented local metastasis. Parotid-gland tissue in the mouse is composed of many spreading nodules and is not so compact as it is in man. These

small nodules at the margin, although apparently separated from the main mass of the tumor, may have developed within peripheral areas of the parotid gland.

The histogenesis of the tumors may be reconstructed as follows: Many different foci appeared within the parotid gland. Initially these were characterized by a loss of acinar structure, often accompanied by the intercellular accumulation of an amorphous hyaline substance. Within these areas, other foci of neoplastic tissue with more vigorous growth capacity developed, and these foci expanded and compressed pre-existing tissue. Some of these more vigorous growth areas still retained an epithelial tubular element. However, a mesenchymal and fibroblastic component represented by fusiform cells showed a greater growth capacity as indicated by numerous mitotic figures. Continuing growth of the sarcoma-like component resulted in large nodules of tumor tissue from which all epithelial elements had disappeared.

The smallest cysts apparently developed from dilatation of the epithelial tubules. This may not apply to the origin of the larger cysts. Some of these may have resulted from necrosis in poorly nourished areas of tumor tissue. However, the fact that cysts were often found in the transplanted tumors indicated that they were an integral feature of the neoplastic growth.

Transplanted tumors.—No morphologic difference was found between the original tumors from which progressive growth on transplantation was obtained, and those which failed to grow (table 3). No attempts were made to transplant very small tumors, and all those successfully transplanted contained large nodules composed of a mesenchymal neoplastic tissue. The transplanted tumors were divided into: (1) type A (including a subtype designated A-1), which was composed of mesenchymal tissue only; (2) type B which contained both epithelial tubular and mesenchymal elements; and (3) type S which included one tumor requiring special description. The A-1 subtype was of special interest, since it was the most frequent pattern observed, being found in tumor lines designated II, VI, XII, and XIV (table 3). Figure 24 shows its characteristic features. Vascular spaces with distinct endothelial linings were rather uniformly distributed throughout the tumor. A frequent feature was a variable degree of lymphocytic infiltration in cells adjacent to the blood vessels. In all A-1 type tumors the neoplastic cells adjacent to the endothelium of the vessels were round and stained blue with hematoxylin, while cells more remote from the vessels were fusiform and eosinophilic (fig. 24). Numerous cysts were interspersed in the tumor tissue at a rather constant distance from the blood vessels. The walls of the cysts were formed by fusiform or flattened cells, which were often necrotic. This pattern, with the change in cell shape from round to fusiform as the distance from the vessel increased was similar to the stratification found in myoepitheliomas. It was also similar to areas sometimes found in the pleomorphic primary tumors. Spontaneous myoepitheliomas, however, have not shown lymphocytic infiltration, and they have large central

cysts, rather than multiple small ones. One transplanted tumor (line III in the second generation) was composed of mesenchymal neoplastic tissue, having a diffuse arrangement of cells, without the distinctive pattern in relation to blood vessels shown by the A-1 type tumors (fig. 25). Two tumors, one in line III in the first generation, and the second in line VIII were designated as type B because they contained many epithelial tubules (figs. 26 and 27). These tumors grew more slowly, and required 17 and 10 months before they were readily palpable. A frond-like or lobular arrangement with a central stalk of paler cells, and more heavily stained cells at the periphery, as in some primary tumors, appeared in line VIII. In the second generation these tumors lost the epithelial component. Line III became an A type, and line VIII a fibrosarcoma. The growth rate accelerated. One tumor designated "S" in line X (fig. 28) required 5 months to reach the size of a "rice grain." It was composed of a single encapsulated nodule and was too small to transfer. Several microscopic cysts were present and the tumor tissue was a mixture of mesenchymal and tubular elements. This tumor mimicked the smaller nodules found in the primary tumors more closely than did any other transplanted tumor.

An extraordinary consequence 10 months following intraperitoneal transplantation from a primary tumor (line XII) was the development of a neoplasm composed of fusiform cells within the parotid-gland area of the recipient mouse. The neoplasm infiltrated and replaced the parotid gland, so that only a few ducts persisted. No intraperitoneal growth was found. A subcutaneous transplant from the same primary tumor to another mouse resulted in a tumor of type A-1 at the transplant site. The tumor occupying the site of the parotid gland is shown in figure 29. It is not possible at the present time to know whether there was any relation between the intraperitoneal injection of tumor tissue and the neoplasm in the parotid. We have not previously seen a tumor of this type in the parotid gland in mice, although fibrosarcomas surrounding mammary ducts are not uncommon.

It appears from this survey that the transplanted tumors reproduce some of the patterns found in the original tumors but the structure of the transplanted tumors was generally uniform throughout, and they did not show the same degree of pleomorphic nodular development. While the original tumors were similar as a group, but varied in different areas in each tumor, the transplants were generally uniform throughout the extent of each tumor. The frequent occurrence of a particular pattern, A-1, among the transplants cannot be explained. The uniform morphology in the transplanted tumors was notably different from the diversity of tissue which developed in a transplanted ovarian teratoma of the mouse (*29*). The teratoma reproduced all the structures found in the original, even though only a small fragment was transplanted. In the parotid-gland tumors the multinodular pleomorphic character of the primary tumors was lost.

No leukemoid reaction in the host has been found from either primary or transplanted tumors.

Comparison with parotid tumors in man.—Certain features of the parotid tumors in the mouse resemble "mixed tumors" of the parotid gland in man. The tumors of the mouse were nodular, multicentric, pleomorphic, and, in early stages, were encapsulated by bands of hyaline or fibrous tissue. The hyaline substance in the mouse resembled the hyaline degeneration found in human tumors, but the hyaline change in the mouse appeared to develop in previously normal tissue during early stages of neoplasia and was not a secondary degeneration of tumor tissue. No cartilage has been observed in the mouse. In human tumors, acini are absent from the fully developed tumors, as in the mouse. Tumors in the mouse, however, unlike the human tumors, have shown no development of epidermoid tissue, and progression of the growth has always been toward a sarcomatous structure, with progressive loss of epithelial elements.

Subcutaneous tumors.—The morphology of most of the subcutaneous tumors was similar to that of other subcutaneous tumors which have been encountered previously in untreated $C3H_f$/He mice. A total of 62 were found in 736 females averaging 18.6 months of age, in which all subcutaneous tumors were examined (*20*). In the tumors from mice in this experiment several showed unusual infiltrative activity. One subcutaneous tumor showed considerable areas of osteogenic activity (fig. 30). Morphologically it differed from the rare osteogenic sarcomas found in strain C3H mice, which have a distinctive pattern (*30*). Two subcutaneous tumors in a hybrid ($C3H_f \times C3H/Fg)F_1$ mouse require special comment (figs. 31 and 32). No parotid tumors were present, but the subcutaneous tumors displayed the distinctive morphology of the tumors originating in the parotid gland of treated mice. This finding cannot now be explained. It may be that subcutaneous glandular elements underwent a similar alteration to that observed in the parotid gland. This is not unlikely when the embryological development of the parotid gland and glands of the skin are considered.

A large neoplasm was found at the site of the left adrenal in a mouse which also had bilateral parotid-gland tumors. It was histologically similar to adrenal neoplasms described by S. Stewart (fig. 33). It was successfully transplanted.

Discussion

It is apparent that there was no increase observed in lymphocytic neoplasms or other forms of reticular neoplasms among 283 $C3H_f$ mice receiving subcutaneous inoculations of various preparations of leukemic tissues. Approximately equal numbers of mice of two sublines, $C3H_f$/Lw and $C3H_f$/He, and a large sample of spontaneous as well as transplantable leukemias, particularly from the AKR and C58 strains, were used in an attempt to corroborate the work of Gross. Five leukemias, 3 spontaneous and 2 early transplantable lines, of the specific subline of the AK strain (AK-n) used by Gross were also studied.

The 3 early leukemic deaths of $C3H_f$ mice in this series, one at 3, and 2 at 7 months, may well be a sampling phenomenon and not related to the

experimental procedure. Although the earliest leukemic death in our control group was at 10 months, leukemic deaths have been obtained at the National Cancer Institute in C3H and $C3H_f$ mice as early as 4 and 5 months of age (*31*).

The early leukemic deaths observed in $(C3H_f \times AKR)F_1$ mice may possibly be related to some leukemia-inducing substance obtained from leukemic tissues. The threshold of sensitivity to induction of leukemia in these mice may be increased by introduction of chromatin from the AKR strain. This possibility is strengthened by the observations of Stewart (*32*), that heating of extracts and filtrates at 80 to 90° C. for 1 hour inactivated the preparations when tested in $(C3H_f \times AKR)F_1$ mice. Certain other experimental procedures, however, also hasten the appearance of leukemia in $(C3H_f \times AKR)F_1$ mice. It has been observed in this laboratory that these F_1 mice develop leukemia spontaneously. In a sample of 165 mice (82 males and 83 females) 56.4 percent died of leukemia at 14.3 months of age, the first leukemic deaths occurring at 9 months. In the present series of controls to group II experimental mice, some leukemic deaths were observed as early as 7 months. Transplantation of thymic fragments from the high-leukemic AKR strain (*7*), intravenous inoculations of intact bone-marrow cells from AKR mice (*33*), or irradiation of $(C3H_f \times AKR)F_1$ mice (*33*), all strikingly increase the probability of early leukemic death.

The possibility of differences in response due to subline differences in the C3H strain remains to be investigated further. Extreme skeletal differences, probably genetic in origin, have been observed among the different sublines of C3H mice (*34—36*). Differences in histocompatibility genes have been detected, at least between 2 sublines and, as pointed out earlier, one subline, C3H/Fg, appears to have a much higher incidence of lymphomas and possibly parotid tumors than others (*10*). Recently, Gross (*37*) has concluded that his data show a difference in response (at least as far as induction of leukemia is concerned) to cell-free materials of the 2 sublines used in his work, the Bittner subline and the Andervont subline (C3H/An), closely related to the sublines used in our studies. According to these recent data the C3H/An subline is strikingly nonsusceptible to filtrates obtained from leukemic tissues, whereas 28 percent of the Bittner subline died from leukemia at 10.8 months of age following inoculations at infancy. It is therefore evident that stringent brother × sister matings should be adhered to in experiments involving the use of C3H mice, and the origin and breeding history fully and completely documented.

Another word of caution is given those interested in studies of leukemia in mice. We have observed many cases of lymphoid hyperplasia, especially in $C3H_f$ mice and $(C3H_f \times AKR)F_1$ hybrids which strikingly simulate, grossly, early lymphocytic neoplasms. It is urged that all suspected cases of leukemia be studied histologically.

A most interesting observation is the occurrence of pleomorphic tumors of the parotid gland, corroborating and extending the findings of Gross

(*1–5*). All but 5 of the 56 tumors observed to date have been bilateral and all but 3 have been found on gross observation. These tumors have grown slowly following transplantation. In a few cases carried serially to date, the mesenchymal or fibroblastic component has been perpetuated. These growths have shown little tendency to invasive growth. It should be re-emphasized that these pleomorphic tumors have not been observed at the National Cancer Institute in any of the several sublines of C3H mice, nor in the high-leukemic AKR and C58 strains, intact, or in those thymectomized to increase life expectancy (*38*). Two spontaneous cases of bilateral tumors at 8 and 10 months have been observed, however, in a small series of C3H/Fg mice in this laboratory. Attention should be called to the high incidence of these neoplasms in the cross involving the C3H/Fg subline $(C3H_f \times C3H/Fg)F_1$ mice.

Parotid-gland neoplasms in this study have appeared in mice receiving inoculations of material prepared from 1) lymphocytic neoplasms obtained from AKR, AK-n, and C58 mice, and of lymphocytic and Hodgkin's-like lesions of C3H/Fg mice; 2) a transplantable neoplasm of the adrenal medulla and from induced parotid neoplasms in $C3H_f$ mice (2 cases). Gross has reported the induction of such neoplasms from preparations of normal tissues of the AK-n high-leukemic strain (*39*). The possibility of the widespread occurrence (at least in nonleukemic strains) of a parotid-tumor-inducing substance has not been investigated.

These studies have been, for the most part, of an exploratory nature; thus attempts have been unsuccessful to find correlations between induction of parotid tumors and 1) pedigreed mother contributing the litter, 2) source of leukemic material used (strain of mouse, spontaneous *versus* transferred leukemia, etc.), and 3) method of preparation of the material (filtrates *versus* centrifugates, etc.). There appears, however, to be a tendency for tumors of the parotid gland to appear in certain litters. Parotid tumors were confined to 15 of the 78 litters comprising groups I, II, and III.

Of interest also are the 10 subcutaneous tumors appearing in the 2 sets of F_1 hybrid mice, particularly in $(C3H_f \times C3H/Fg)F_1$ hybrids. These were found, in all but one case, in mice of both sexes having bilateral parotid neoplasms. They resemble histologically the subcutaneous sarcomas found, upon occasion, in old mice of the C3H strain. These growths were found as early as 3 and as late as 12 months, and none has been observed among the litter controls. The appearance of 3 subcutaneous sarcomas among 283 $C3H_f$ mice in the experimental series and 2 in 161 controls of group I would appear to have no significance since these appeared late in life, beyond 15 months. Experiments are now in progress to determine the significance of these findings. It should be recognized that C3H strain mice are indeed especially sensitive to the induction of subcutaneous sarcomas by carcinogenic hydrocarbons (*40*) and that hybridization, at least as studied in certain F_1, F_2 and backcross mice (*41*) gives rise to many abnormalities and morphologic forms of neoplasms not encountered in the parental strains.

Hepatomas and mammary tumors were found about equally distributed in the experimental and control groups, especially in group I, $C3H_f$ mice. These mice have attained the highest average age and almost all have succumbed. Granulosa-cell tumors of the ovary and adrenal cortical adenomas were next in frequency of occurrence. Three carcinomas of the adrenal medulla were found among the experimental mice but one was also found in the group I control series but in an older animal (19 months of age). These were similar morphologically to those observed by Stewart, who favors the assumption of a relationship between these tumors and the inoculation of leukemic material (*32*).

The observations recorded by Gross (*1–5*), by Stewart (*32*), and those discussed here are, of necessity, pilot experiments. All are totally inadequate to establish a direct relationship between one or more cell-free agents and specific morphologic forms of neoplasms.

Summary

Twenty-two (7.7 percent) pleomorphic tumors of parotid-gland origin have been observed to date among 283 $C3H_f$ mice inoculated, when less than 24 hours of age, with preparations of leukemic tissues obtained from the high-leukemic strains AKR and C58. The average host age at appearance of these tumors was 11.7 months and the age ranges were from 4 to 19 months. The tumors were confined to 8 of 55 litters, and in 5 litters all sibs had tumors of the parotid gland.

Transplantation of the parotid tumors in $C3H_f$ mice was accomplished in only 7 of 15 tumors attempted.

Nine tumors of parotid-gland origin have been observed among 113 $(C3H_f \times AKR)F_1$ mice. These have occurred in 5 of 17 litters, in mice from 3 to 12 months of age, following inoculations of preparations from AKR, AK-n, and C3H/Fg leukemias. Five of 21 leukemic deaths in this experimental group were found at 4 to 8 months, before the appearance of leukemia among control $(C3H_f \times AKR)F_1$ mice. The incidence of leukemia among control mice at 18 months, however, is strikingly higher than that observed in the inoculated group. Two mice of the experimental group had both lymphocytic neoplasms and bilateral tumors of the parotid gland while 2 others had subcutaneous growths and parotid tumors.

Four of 5 transplanted lymphocytic neoplasms but only 1 of 7 parotid tumors grew progressively in $(C3H_f \times AKR)F_1$ mice. They did not, however, grow progressively in mice used as donors of leukemic tissue, the AKR, AK-n, and C3H/Fg strains.

In a small series of 28 $(C3H_f \times C3H/Fg)F_1$ mice, 10 have presented tumors of the parotid gland at from 5 to 12 months of age following inoculations, into infant mice less than 24 hours old, of leukemic preparations from C3H/Fg and C58 mice. Eight of these mice had, in addition, subcutaneous neoplasms. One mouse had a subcutaneous neoplasm only. These neoplasms were observed in only 3 of 6 litters. They occurred about equally in males and females. Among uninoculated ($C3H_f \times$

C3H/Fg)F_1 control mice, only one neoplasm, a lymphocytic neoplasm, has been found to date.

Four of 5 subcutaneous neoplasms have grown progressively, and through at least 3 serial passages, only in the specific F_1 mice used in this experimental group.

Thirty-three offspring constituting the F_1 and F_2 generations from 2 $C3H_f$ females which had bilateral parotid-gland tumors during the period of nursing have not developed these neoplasms.

A description of the morphology of pleomorphic tumors of the parotid gland in the 3 groups of experimental animals is given.

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All sections stained by hematoxylin and eosin unless otherwise stated. Further information on mice from which sections were taken is given in the tables.

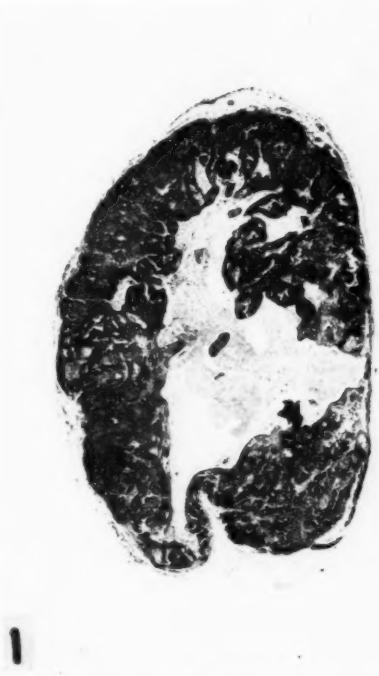
PLATE 32

FIGURE 1.—Myoepithelioma. Entire cross section of a primary tumor. Note central cyst and uniform pattern throughout the section. × 8 (strain A/Hen, female, 5 months)

FIGURE 2.—Higher magnification of myoepithelioma. The cells of the tumor are roughly stratified, or arranged in layers. A round or oval cell occupies a basal position next to bands of connective tissue which form the stroma of the tumor. Mitoses are frequent in this layer of cells. Cells lying above the basal layer are fusiform, and the more centrally located cells are flattened and often necrotic. × 290

FIGURE 3.—Bilateral, multinodular tumors of the parotid gland. × 2 (table 2, expt. 57, female, 5 months)

FIGURE 4.—Entire cross section of a tumor of the parotid gland, showing several characteristic features. Large nodule at top is composed of a mixture of epithelial tubules and mesenchymal tissue, with central cysts. Walls of these cysts appear ragged and necrotic. Normal parotid-gland tissue remains in *center* and surrounds expanding neoplasm in *lower left*. A cervical node on *right* is not invaded by tumor. Confluent nodules of tumor tissue in *lower center* contain a cyst lined by flattened cells. A mass of hyaline material in *lower left*. Lowermost nodule, adjoining normal tissue, shows early neoplastic change with disappearance of acini. × 21 (table 2, expt. 71, male, 7 months)



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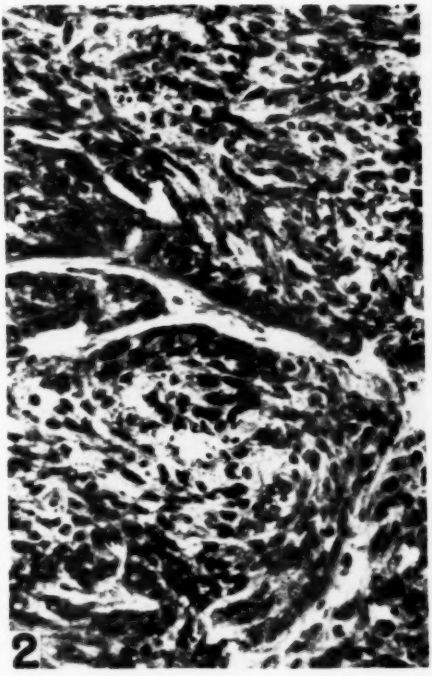

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PLATE 33

FIGURE 5.—Entire cross section of a tumor of the parotid gland, showing several characteristic features. A series of photographs taken at high magnification was made from different areas of this tumor. Some normal parotid-gland tissue and an atrophic lymph node are seen on the left. Nodules of tumor tissue extend into mammary gland areas, recognizable by mammary ducts. × 16 (table 2, expt. 54, female, 16 months)

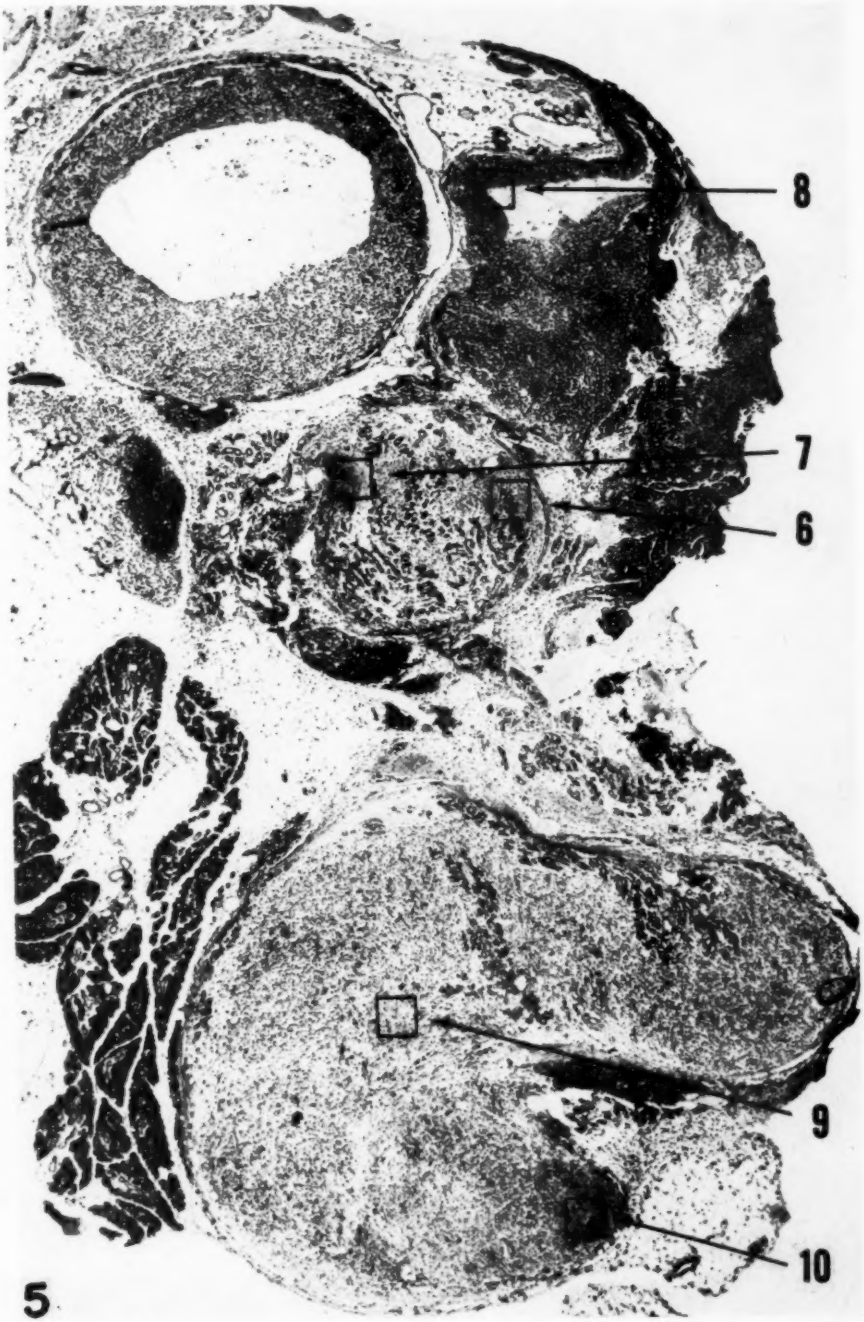

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PLATE 34

FIGURE 6.—Higher magnification from area in figure 5. This shows characteristic arrangement of small epithelial tubules lined by cuboidal epithelium. A cellular, mesenchymal type of tissue composed of fusiform cells lies between the tubules. × 340

FIGURE 7.—An accumulation of hyaline substance lying at the margin of a nodule in figure 5. Material at *top* is denser, less cellular, and more eosinophilic than in *lower* part of photograph. Persisting tubular structures can be seen at *lower right*. × 340

FIGURE 8.—Margin of a cyst shown in figure 5. Surrounding tumor tissue is unusually cellular and hematoxylinophilic, resembling fetal connective tissue. Cyst wall is composed of flattened cells. × 340

FIGURE 9.—Area from tumor in figure 5, showing more differentiated type of fibroblastic cells, with collagen formation. This degree of differentiation was infrequent in parotid-gland neoplasms. × 340

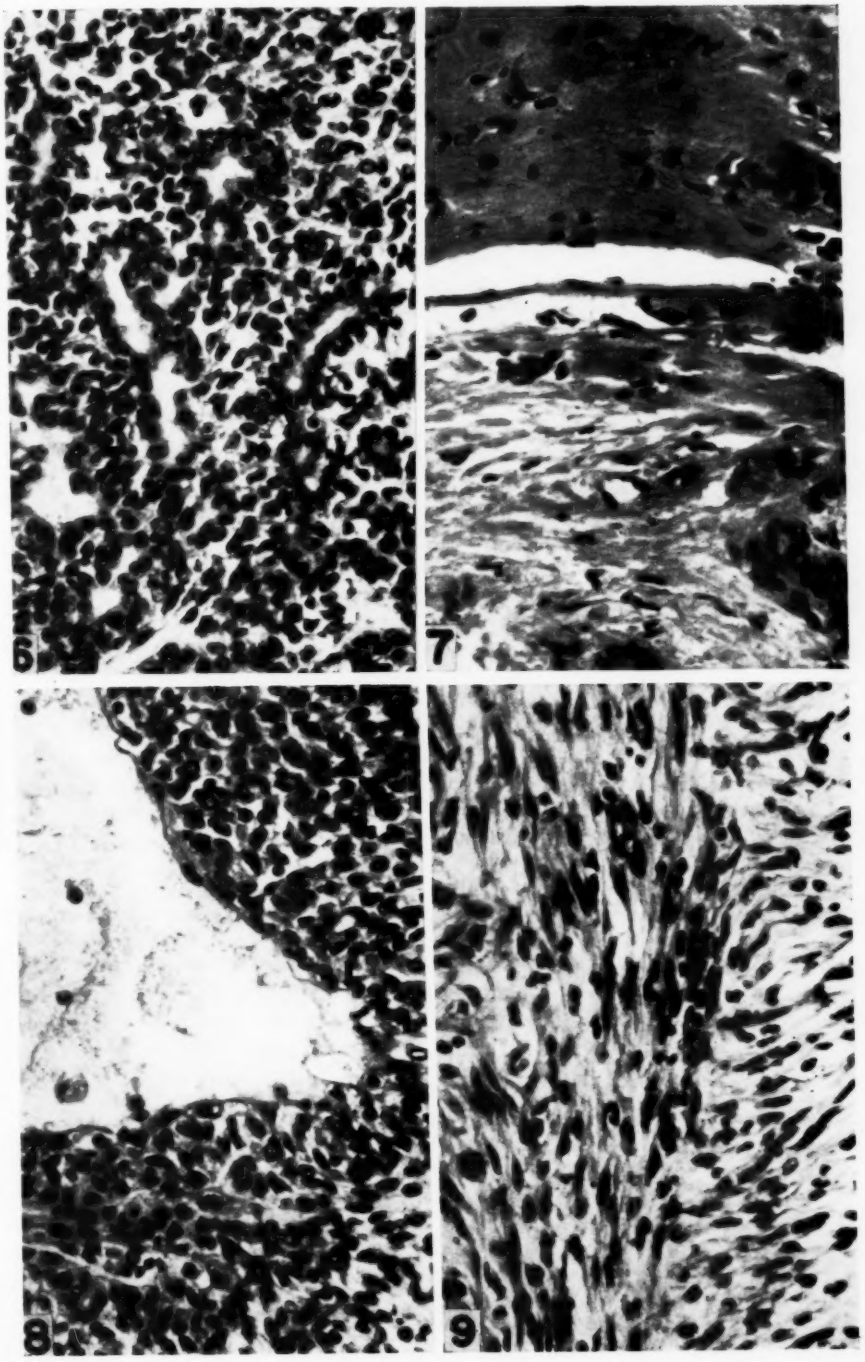
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PLATE 35

FIGURE 10.—Area from tumor in figure 5. Areas with heavy lymphocytic infiltration, usually concentrated about blood vessels, are common. × 340

FIGURE 11.—A frond-like or lobular pattern developing in a nodule from a large tumor. Cells are more densely packed at the margin. In the interior, the cell nuclei are loosely arranged and separated by delicate fibrils with vacuoles between. × 340 (table 2, expt. 8, male, 19 months)

FIGURE 12.—Another common pattern in which there is alternation of densely packed and loosely packed cells. More cellular areas are found around the blood vessels. Cells distant from the blood vessels are sometimes fusiform as in *lower right*, and this pattern resembles the stratification found in the myoepitheliomas. × 340 (table 2, expt. 63, male, 16 months)

FIGURE 13.—Lacy pattern frequently found. Spaces are filled with fluid which was sometimes stained deeply with hematoxylin but was not metachromatic with toluidine blue. × 340 (table 2, expt. 57, female, 5 months)

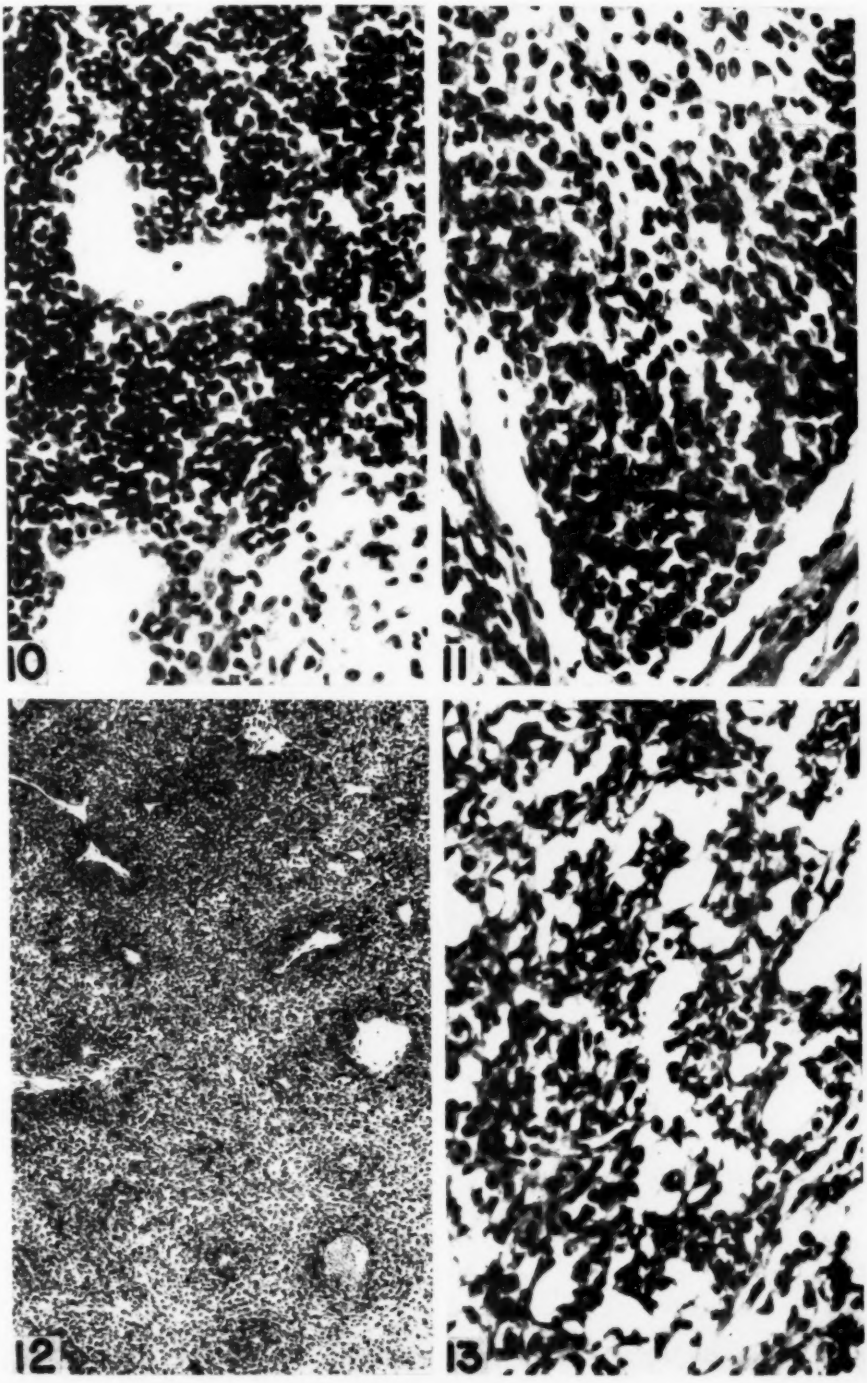
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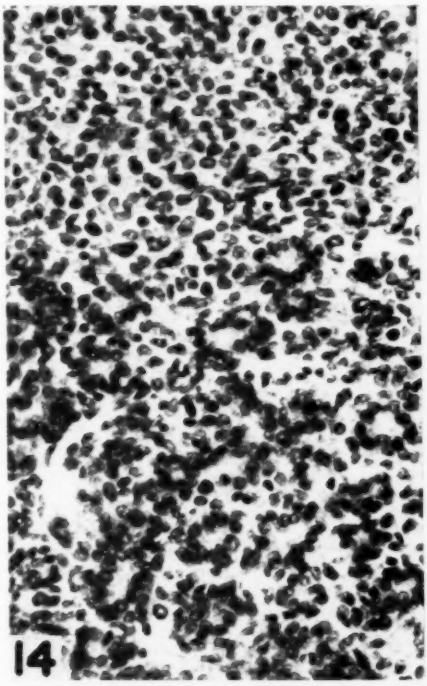
PLATE 36

FIGURE 14.—An area where epithelial tubular structures merge with an area composed entirely of oval cells without glandular formations. × 290 (table 2, expt. 54, male, 14 months)

FIGURE 15.—Skin area overlying a parotid-gland tumor. Skin is infiltrated by neoplastic fusiform cells with abundant collagen. Tissue resembles differentiated fibroblastic tissue seen in figure 9. × 65 (table 2, expt. 63, male, 6 months)

FIGURE 16.—The smallest tumor so far observed, found upon histologic examination. All acini have disappeared, and multicentric foci, some of which are circled, show rapid growth and compression of surrounding tissue. In the largest nodule tubular structures are disappearing from the center and being pushed to the margin by a growth of fusiform cells. Several small cysts are lined by cuboidal epithelium. Pale, non-cellular areas represent hyaline deposits. The overlying muscle is pushed outward but not invaded by the expanding tumor. Remaining parotid-gland tissue appears normal. × 65 (table 5, expt. 32, male, 9 months)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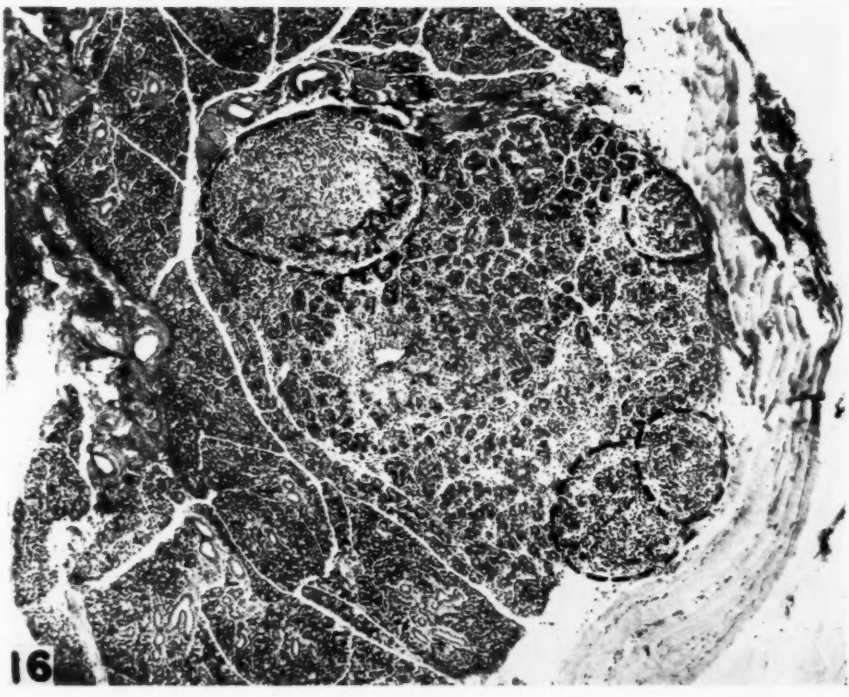
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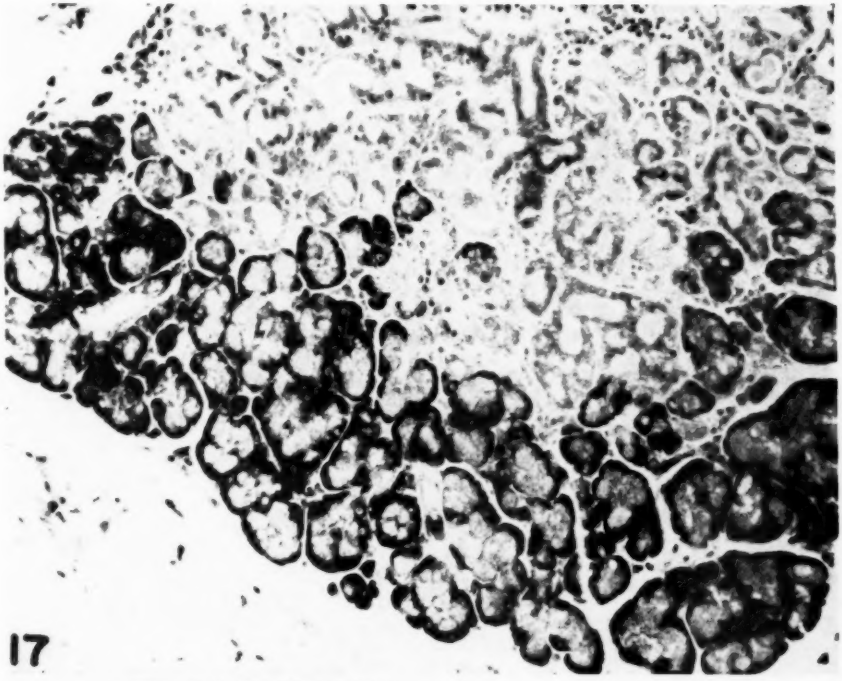
PLATE 37

FIGURE 17.—Area from the exorbital lachrymal gland in an animal which had a large parotid-gland tumor. The normal acini are being replaced by epithelial tubules. This disappearance of acini is associated with the neoplastic change. Toluidine blue. × 230 (table 2, expt. 57, female, 5 months)

FIGURE 18.—Periodic acid-Schiff preparation of same area. Tubular structures are filled by a Schiff-positive material. × 275

FIGURE 19.—Rare instance of involvement of the male submaxillary gland in the neoplastic process. More highly differentiated acinar and ductular structures are replaced by tubules formed of low cuboidal cells. × 65 (table 2, expt. 63, male, 6 months)



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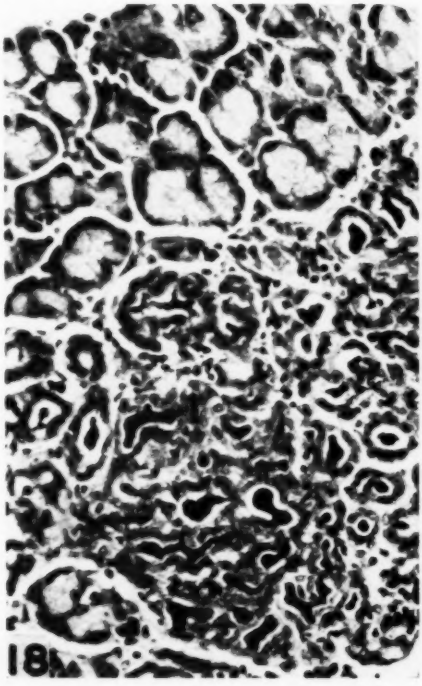

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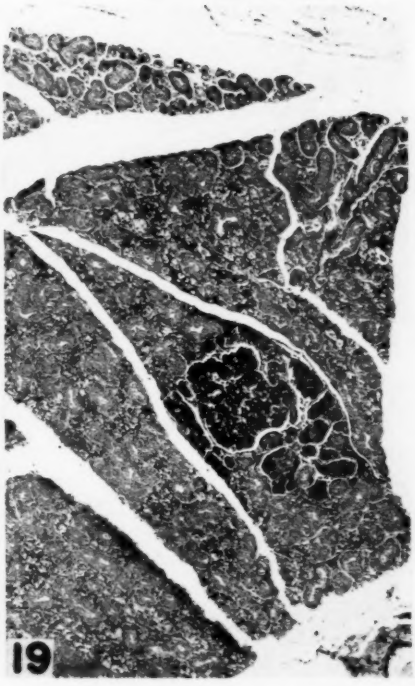

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PLATE 38

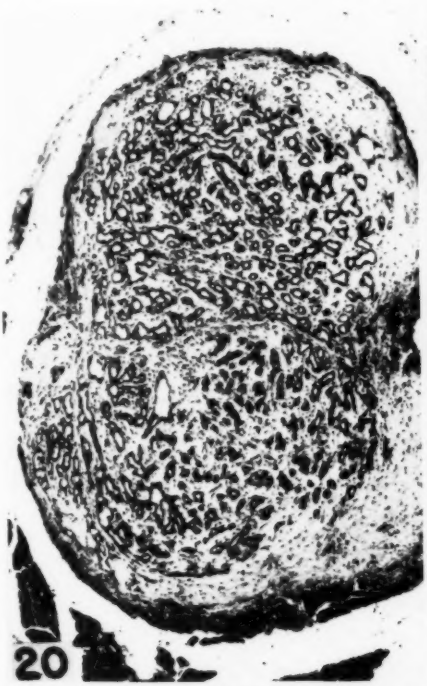
Progressive development in hyaline nodules

FIGURE 20.—Nodule composed of small tubules and cysts separated by an abundant hyaline material in which a few oval nuclei are found. × 80 (table 2, expt. 90, male, 10 months)

FIGURE 21.—Nodule in which proliferation of a tissue composed of a cellular stroma and numerous small tubules appears to be developing centrally and pushing the hyaline substance peripherally. × 75 (table 2, expt. 57, female, 5 months)

FIGURE 22.—Margins of three nodules surrounded by bands of hyaline substance remaining at the margins of the expanding nodules. × 100 (table 2, expt. 57, 5 months)

FIGURE 23.—Reticulum fibers concentrated at the margins of two adjacent nodules. Reticulum fibers were sparse within tumor tissue but abundant at the margins of growth and in the normal parotid-gland stroma. Wilder method for reticulum. × 100 (table 2, expt. 57, male, 5 months)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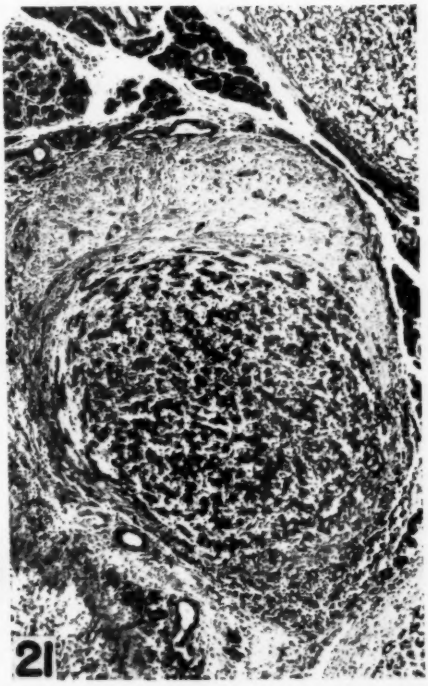
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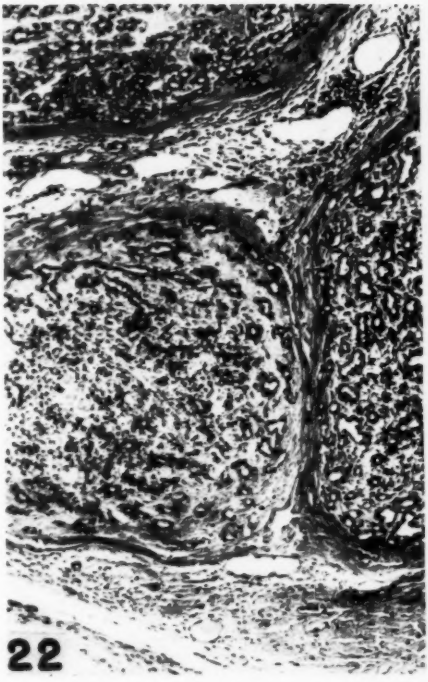
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PLATE 39

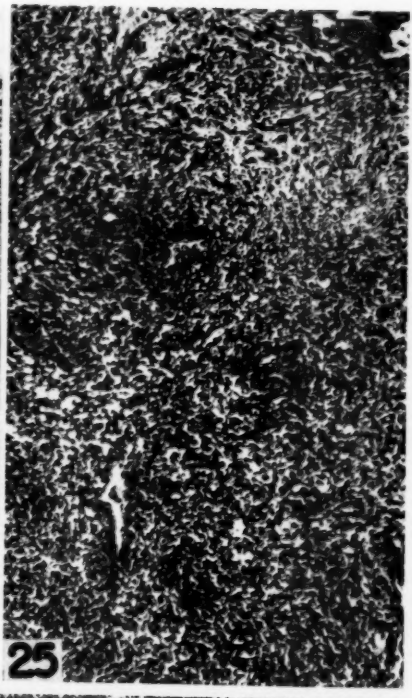
Transplanted tumors developing from parotid-gland tumors. (Figs. 24–28)

FIGURE 24.—Type A-1. The most commonly occurring pattern. Cells adjacent to blood vessels are round, basophilic, and densely packed. In the example shown here, an infiltration of normal-appearing lymphocytes is found among the perivascular cells. Cells distant from the vessels are fusiform and less densely crowded. Cysts lined by fusiform cells are regularly interspersed in areas distant from the vessels. × 65 (table 3, expt. 8, II)

FIGURE 25.—Type A. A transplanted tumor having no epithelial structures and lacking the regular pattern of figure 24. × 100 (table 3, expt. 58, XII)

FIGURE 26.—Type B. Transplanted tumor showing a frond-like lobular pattern. Lobules are partly surrounded by connective-tissue stroma. Central area in a lobule is composed of pale-staining cells, often necrotic. Epithelial tubules are seen in peripheral area, separated by a mesenchymal type of tissue. × 80 (table 3, expt. 57, VIII)

24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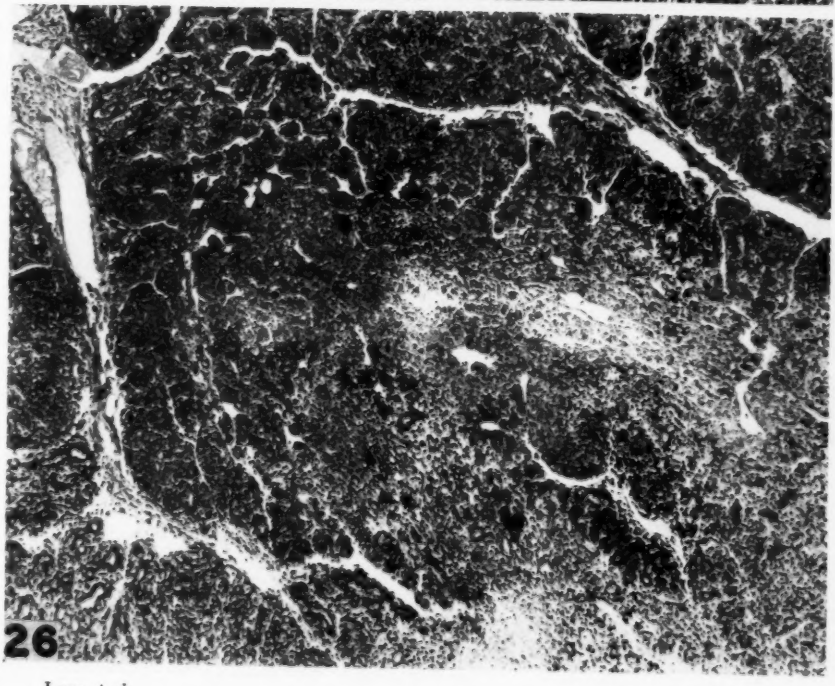
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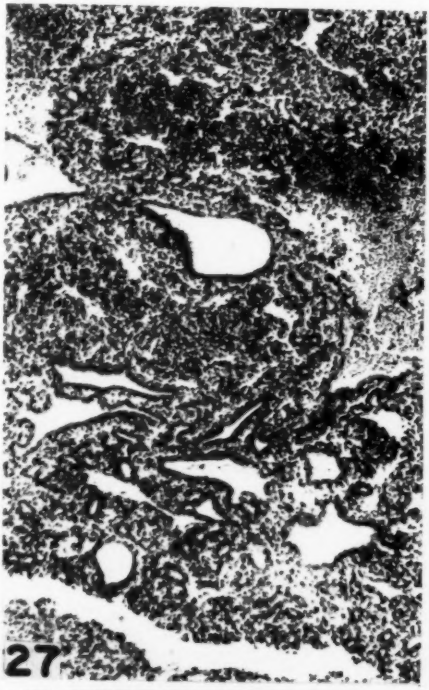
PLATE 40

FIGURE 27.—Type B. Transplant in which tubules and spaces lined by cuboidal epithelium are frequent. × 100 (table 3, expt. 8, III)

FIGURE 28.—Type S. Entire cross section of a tumor which required 5 months to reach this size. Characteristic features of the primary tumor still retained are the pattern of epithelial tubules separated by fusiform cells, nodular growth with encapsulation, and cysts. × 33 (table 3, expt. 57, X)

FIGURE 29.—Tumor occupying site of parotid gland in a mouse given an intraperitoneal injection of a small bit of tissue from a parotid-gland tumor 10 months previously. Present tumor composed of fusiform fibroblastic cells, with variable degree of collagen formation. Two nodular areas of neoplastic fusiform cells are surrounded by concentric layers of hyalinized tissue. × 100 (table 3, expt. 58, XII)

FIGURE 30.—Tumor with scalloped margin showing bone formation in center. × 60 (table 5, expt. GH-21, female, 8 months)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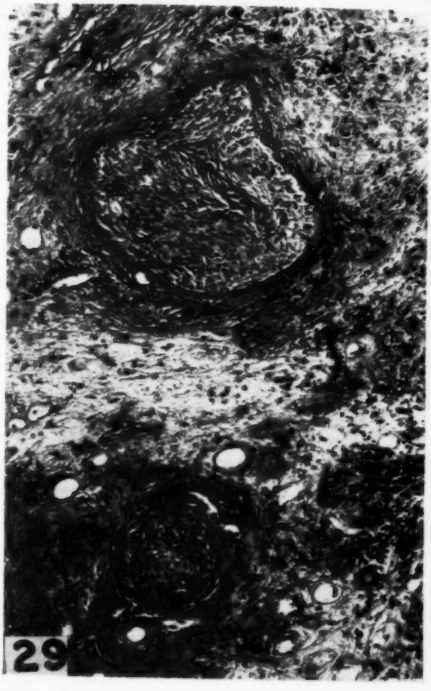
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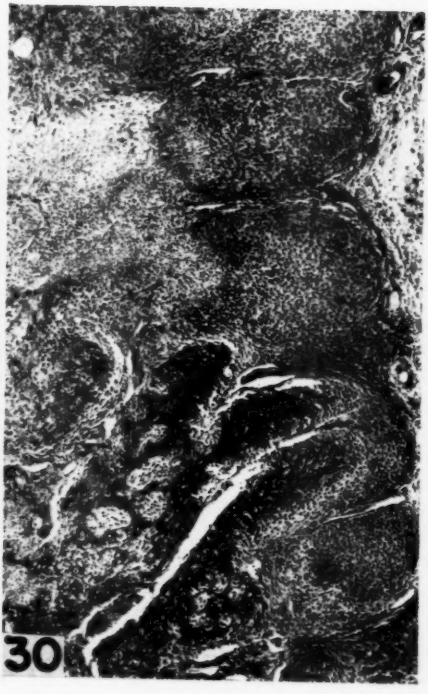
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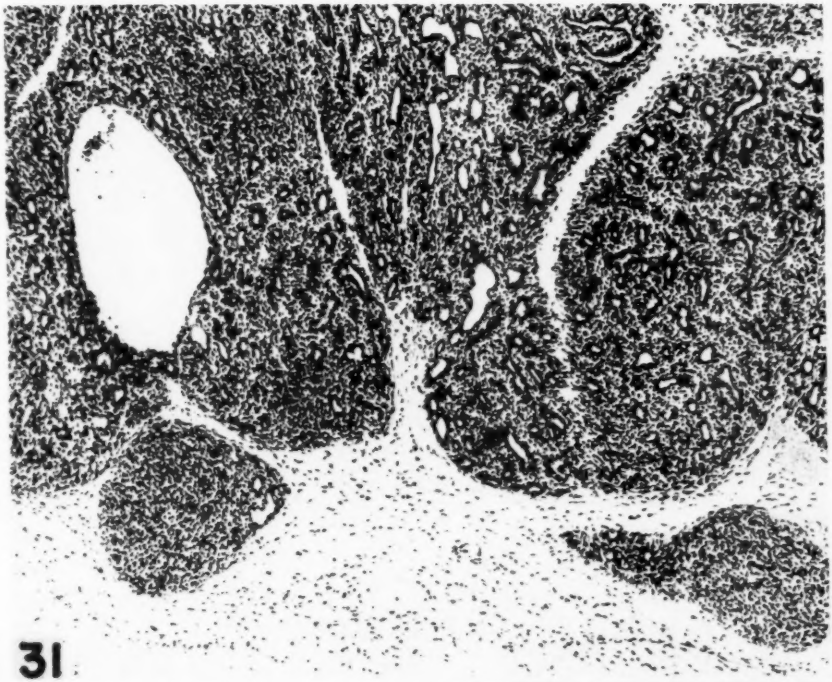
PLATE 41

FIGURES 31 and 32.—Two separate subcutaneous tumors found in the dorsal area of a treated mouse. These tumors reproduce patterns commonly found in primary and transplanted parotid-gland tumors. Parotid glands in this mouse were normal. × 270 (table 5, expt. GH-26, male, 3 months)

FIGURE 33.—Tumor found at the site of the adrenal gland and replacing it. × 270 (table 2, expt. 57, female, 4 months)



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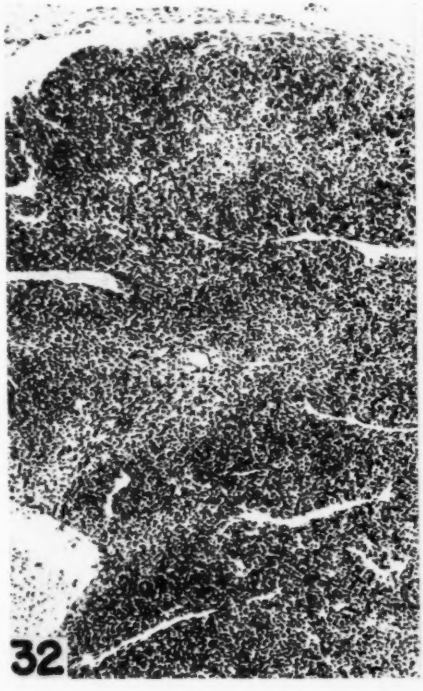

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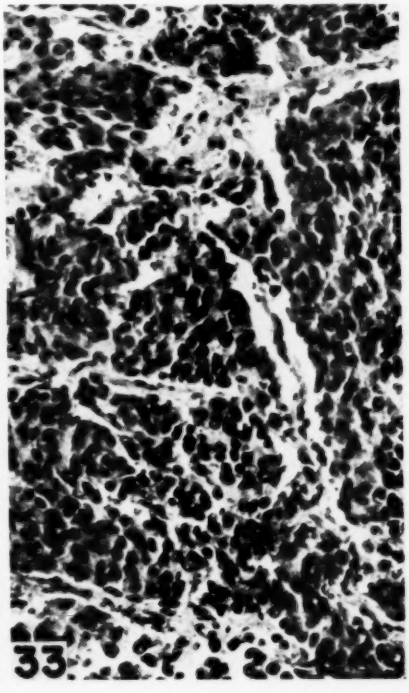

33

The *p*H of Rat Tumors Measured *In Vivo*[1]

M. EDEN, B. HAINES, *and* H. KAHLER,[2] *National Cancer Institute,*[3] *Bethesda, Md.*

Introduction

Following the observation (*1*) that tumor slices exhibit an increase in lactic acid concentration when glucose is offered as a metabolite, an investigation was started on the *p*H determination of normal and malignant tissue by means of the glass electrode (*2–5*). The main results of this early work may be summarized as follows: 1) Normal muscle and liver had a *p*H similar to that of blood, namely, around 7.4. 2) Rat tumors had a *p*H considerably lower, about 7.0 3) After intraperitoneal injection of massive doses of glucose the *p*H of tumors dropped approximately half a *p*H unit to *p*H 6.4–6.5, whereas normal tissue remained relatively unchanged. 4) After a period of several hours, the *p*H of the tumor stabilized at approximately the starting value. 5) Certain sugars, such as lactose and galactose, produced no significant change in the *p*H of the Jensen sarcoma or Flexner-Jobling carcinoma.

It was pointed out in these investigations that the glass electrode used, the effective contact surface of which was in the form of a capillary spicule, unavoidably caused tissue injury but probably measured the *p*H of the extracellular fluid.

More recent investigations on this subject carried out by others include measurements on rats, mice, and human beings. The *p*H of human tumors was found to be lower than that of normal human tissue (*6*). A *p*H drop of about 0.5 units was found in 12 mice with induced tumors after giving carbohydrate, while lower *p*H values were found in 5 cases of human genital cancer as compared to normal tissue (*7*). Consistently lower values were found for many more rat tumors than for normal tissue, while the drop in *p*H following glucose injections seemed to depend upon the particular kind of tumor under investigation (*8*, *9*). A number of

[1] Received for publication May 12, 1955.

[2] We are indebted to Mr. N. F. Coffey for help in the building and maintainance of the recording equipment and to Mr. B. J. Lloyd, Jr. for a search of the bibliography on this subject. Tumors and animals were contributed by Drs. M. K. Barrett, W. E. Heston, M. E. Maver, and H. P. Morris. The glass electrodes were made by Mr. E. J. LeBrun.

[3] National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare.

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other investigations (*10–18*) have been conducted on closely related subjects with different techniques from those we have utilized.

These studies indicated considerable variability in the measurements. Any further progress requires optimal technical procedures and the use of larger numbers of animals for statistical evaluation of the results.

Experimental Procedures

Glass electrodes were employed as the *p*H-sensitive elements. The construction of the electrodes described by Kahler and Robertson (*5*) was modified slightly. The spicular tip was made somewhat shorter, approximately 5 mm., and somewhat narrower, diameter at the base about 1 mm. The over-all length of the body of the electrode was decreased to about 5 cm. The internal electrode was a silver-silver chloride electrode prepared by brazing a heavy silver wire approximately 3 cm. long to a brass cap, leaving about 5 mm. of the silver wire extending outside the cap. The silver wire was heavily chloridized in 0.5 *N* HCl using a current of 5 Ma. for about 30 minutes. The body of the electrode was filled with a solution of 0.025 molar phosphate buffer, *p*H 6.8 and about 0.015 *N*, in chloride ion (KCl). The cap was placed over the top of the glass electrode and sealed with Apiezon wax.

The basic electrical circuit differed only slightly from that described by Kahler and Robertson (*5*). No agar bridge was used; instead, a capillary-tipped calomel half cell was immersed in the Ringer's solution in which the rat's tail had been immersed.[4]

A number of different high-resistance-potential measuring devices were employed at one time or another during the course of the experiments. We found the most convenient and most stable amplifier for use in this application to be the Applied Physics Laboratory vibrating-reed electrometer. Its output was fed into a Brown multiple-point strip-chart recording potentiometer. The sensitivity of the recorder was adjusted so that full-scale deflection was equivalent to 1.00 *p*H unit.

Eleven glass electrodes were monitored by means of two Beckman 6-point automatic switches in conjunction with the recorder and amplifier. The calomel electrode was common to all glass electrodes. Extreme care was required to insure electrical shielding of the glass electrodes from external fields and also to prevent capacitive coupling between the several lines connecting the glass electrodes to the amplifier.

The glass electrodes were calibrated with phosphate buffer (*p*H 6.85) (*19*) and potassium acid phthalate buffer (*p*H 4.01) (*20*) at 25° C. Electrodes were discarded if the *p*H difference observed between these two buffers was less than 2.70 or greater than 2.90 *p*H units.

The temperature sensitivity of the glass electrodes was estimated by immersing the tip of each electrode in phosphate-buffer solutions maintained at definite temperatures and measuring the change in *p*H for a

[4] It has been pointed out in previous publications (*3, 4*) that the potential across the intact skin using Ringer's solution usually amounts to less than 5 mv.

temperature change from 20 to 40° C. So long as the body of the electrode was thermally shielded from the solution, a *p*H change of only 0.05 was observed for this temperature range. Such a change can be attributed to the change in *p*H of the buffer solution from 6.88 at 20° C. to 6.83 at 40° C (*19*).

The recorder print point for each electrode was adjusted so that *p*H 6.85 was registered at the mid-line of the chart. Each electrode was monitored by the recorder every 4 minutes and printed so that consecutive points were displaced 0.067 inch on the time axis. In a small number of experiments the readings were erratic, and no smooth curve could be drawn through the printed points. Such experiments were discarded.

The animal-handling techniques were similar to those described by Kahler and Robertson (*5*), with only minor modifications. Whereas in the former experiments the rats were starved from 18 to 48 hours before measurements were started, in the present work the animals were fed their normal diet up to the time of the experiment. In most of the experiments, the animals were anesthetized with 1 ml. of a 10 percent solution of ethyl urethan[5] injected intraperitoneally for each 100 gm. of body weight, after which they were tied down in a supine position to an insulated board. The tails were then inserted into a glass manifold containing Ringer's solution, and small cutaneous incisions were made in the tumor surface at the point where the electrode was to be inserted. A half-inch mesh screen was placed about 3 cm. above the rats, and the glass electrodes inserted through the screen interstices into the tumor through the incision. In this way the electrodes were held vertical, most of the weight of the electrode was supported by the screen, and small movements of the rat failed to shift the electrode from its site or to break off the spicule.

An attempt was made to use tumors of approximately the same size in the *p*H determinations. This was not entirely successful since two tumors in the same host (rat) sometimes grew at very different rates. We found the optimal size of tumors for *p*H measurement to be about 10 to 15 mm. in diameter; however, we have included tumors as large as 25 mm. in diameter in our series. In such cases, we attempted to position the electrodes close to the margin of the tumor so as to avoid any possible necrosis in the center of the growing mass. Attempts to make measurements with smaller tumors have not been consistently successful since a portion of the glass electrode always protruded into normal tissue and, consequently, influenced the resulting determination.

The rat on the insulated board was transferred to an electrically screened and grounded cage and the electrode leads were connected to the measuring circuit. Within 30 minutes from the onset of anesthesia, the preparation was complete and the recording system turned on. About an hour was

[5] No difference in *p*H registration was seen between urethan and three barbiturates. Since the dosage of the barbiturates was found to be more critical than the dosage of urethan and more frequent supplementary doses were required, the use of barbiturates was abandoned.

permitted to elapse before any further treatment of the test animals, because the electrodes exhibited erratic responses immediately after their insertion into the test animal. Stability was considered to be reached when the slope of the jointed graph indicated a drift of less than 0.03 *p*H unit per hour. The average time from the start of recording to reasonable stability was 30 minutes. After the initial period, a dose of 600 mg. of glucose (as a 20% solution) for each 100 gm. of body weight was injected into each rat.

The elapsed time for each experiment was recorded from 18 to 30 hours, during which time the rats were disturbed as little as possible. Usually a supplementary anesthetic dose was required after about 6 hours. After completion of an experiment the electrodes were removed from the tissue sites, rinsed with distilled water and phosphate buffer, and returned to *p*H 6.85 phosphate-buffer solution. If the electrodes failed to return to the initial set point ± 0.05 *p*H unit, the record for that electrode was discarded. The majority of electrodes returned to the set point within ± 0.03 *p*H unit.

Eight different transplantable rat tumors (*21*) were studied by this procedure:

1) *Lymphosarcoma (J. B. Murphy)*.[6]—This tumor was initially induced by 1,2,5,6-dibenzanthracene and has two modes of growth depending on the method of transplantation. Intraperitoneal inoculation generated acute lymphocytic leukemia, killing in 6 to 10 days. When transplants were made subcutaneously or intramuscularly, massive tumors developed containing many round cells and little stroma. Initially, localized tumors killed the animal in 6 weeks to 2 months (*22*); however, the virulence has increased with time, and, in our experiments with the tumor, death followed after 9 to 14 days in the absence of regression.

We carried this tumor in both the Wistar-Carworth strain and the Sprague-Dawley strain. Behavior in both strains was essentially the same.

2) *Sarcoma 1643 (H. P. Morris)*.—This tumor was initially induced in 1946 with 2-diacetylaminofluorene in the Buffalo-strain rat (*23*). Originally, it was considered to be a mammary carcinoma with acinus formation, but it has transformed during the series of transplantations from a distinctly epithelial structure to a type resembling sarcoma. This is a relatively slow-growing tumor, killing the host in about 70 days.

3) *Sarcoma E2730 (W. E. Heston)*.—This tumor arose spontaneously in 1947 in the sternum of an inbred rat of the Marshall 520 strain. It is a very slow-growing tumor, killing the host in 2 months or more.

4) *Harderian-gland carcinoma 2226 (H. P. Morris)*.—This tumor was originally induced in 1945 with 2-diacetylaminofluorene in the posterior region of the orbital cavity of rats of the Buffalo and Marshall strains (*24*). The tumor grows rapidly upon transplantation, killing the host in 28 days.

5) *Hepatoma N (A. B. Novikoff)*.—A liver tumor induced in 1951 with

[6] The names in parentheses refer to the investigators in whose laboratories the tumors were first observed.

p-dimethylaminoazobenzene in Sprague-Dawley-Holtzman rats. In our laboratory it has been slow-growing when inoculated intramuscularly or subcutaneously, the host surviving for more than 2 months. A brief enzymatic and cytologic study has been reported on this tumor (*24*).

6) *Hepatoma 3924A* (*H. P. Morris*).—Induced with 2-diacetylaminofluorene and hormones in 1951 in the liver of an A × C/9935 (Irish) rat, it grows at a moderate rate, killing the host in 30 to 45 days.

7) *Sarcoma 3741* (*H. P. Morris*).—Induced with 2-diacetylaminofluorene in a Buffalo rat in 1951, it appeared initially as a sarcoma involving the cervix, corpus body of the uterus, and bladder (*25*). This tumor kills the host in 4 to 6 weeks.

8) *Fibrosarcoma ACMCA2* (*W. F. Dunning*). This tumor was first induced in 1946 by the subcutaneous injection of 20-methylcholanthrene in a rat of the A × C/9935 (Irish) strain (*26*). It is a rapidly growing tumor, killing in 30 days.

Experimental Results

The experimental observations on 156 tumor-bearing rats and 15 normal rats are summarized in tables 1 and 2. A typical chart is reproduced in text-figure 1. This chart presents measurements involving 6 electrodes in 1 tumor. The data for this chart are summarized under the heading 3741–9A in tables 1 and 2. A composite representation of the averages for all the electrodes (338) is reproduced in text-figure 2.

It will be noted in table 2 that the number of electrodes used in computing averages has also been given. The experimental observations

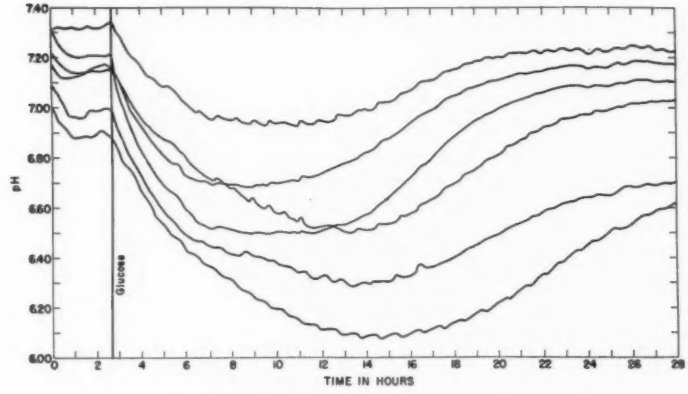
TABLE 1.—*Summary of* p*H response characteristics of various tumor types to intraperitoneal glucose injections (part 1)*

Tumors	Number of rats	Electrodes	*p*H glucose injection	Standard* deviation	Standard* error	*p*H minimum	Standard* deviation	Standard* error
Hepatoma N	21	39	6.96	.17	$.03_0$	6.46	.22	$.03_4$
3741	10	28	6.95	.25	$.04_8$	6.55	.27	$.05_1$
(Rat 9A)	1	6	7.13	.16	$.06_5$	6.51	.28	$.11_5$
(Rat 10A)	1	6	7.09	.07	$.02_7$	6.70	.11	$.04_5$
Lymphosarcoma								
Wistar	25	46	6.98	.20	$.02_9$	6.49	.29	$.04_2$
Sprague-Dawley	20	51	7.03	.19	$.02_7$	6.51	.32	$.04_5$
All Lymphosarcomas	45	97	7.00_5			6.50_1		
E-2730	9	17	7.04	.11	$.03_0$	6.67	.14	$.03_5$
2226	15	32	7.00	.11	$.01_9$	6.54	.23	$.04_1$
1643	23	55	7.01	.16	$.02_1$	6.63	.22	$.03_0$
ACMCA2	15	27	6.83	.24	$.04_7$	6.48	.28	$.05_5$
3924A	16	43	7.06	.22	$.03_4$	6.62	.27	$.04_9$
All Tumors	156	338	6.99_4			6.54_8		
Normal rat muscle	15	29	7.39_6	$.12_2$	$.02_3$	7.25_8	$.12_5$	$.02_3$
Normal liver (5)	12	12	7.39	—	—	—	—	—
Hepatic tumor (5)	10	10	6.99	—	—	6.42	—	—

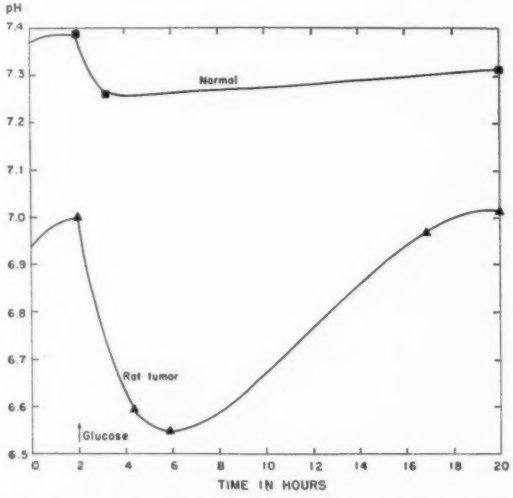
*Computed for all electrodes in each tumor.

TABLE 2.—*Summary of pH response characteristics of various tumor types to intraperitoneal glucose injections (part 2)*

	Time to 90% minimum		Time to minimum		Time to 90% recovery		Maximum drop rate		Maximum recovery rate		*p*H at end of run	
	Electrodes	Hours	Electrodes	Hours	Electrodes	Hours	Electrodes	*p*H units/hr.	Electrodes	*p*H units/hr.	Electrodes	*p*H
Hepatoma N	7	4. 80	32	8. 02	7	15. 35	38	. 22	11	$.06_5$	11	6. 96
3741	17	3. 33	26	4. 15	17	16. 84	27	. 34	23	$.07_1$	18	6. 94
(Rat 9A)	6	5. 72	6	7. 17	5	19. 9	6	. 19	6	$.06_2$	5	7. 06
Lymphosarcoma												
Wistar	37	2. 22	46	3. 87	36	16. 08	46	. 40	44	$.10_5$	46	7. 03
Sprague-Dawley	43	1. 60	51	3. 07	46	15. 84	51	. 54	51	$.10_1$	50	7. 11
E-2730	—	—	17	2. 48	—	—	—	—	—	—	—	—
2226	—	—	36	3. 35	—	—	—	—	—	—	—	—
1643	5	3. 93	52	3. 35	7	11. 82	7	. 40	7	$.14_7$	7	7. 18
ACMCA2	22	1. 75	26	3. 48	16	12. 23	27	. 36	22	$.09_5$	22	6. 86
3924A	27	2. 69	36	3. 40	30	13. 06	36	. 37	29	$.09_1$	30	6. 99
All Tumors	165	2. 33	327	3. 87	163	14. 89	238	$.38_7$	191	$.09_5$	190	7.01_2
Normal rat muscle	—	—	13	1. 20	—	—	15	$.17_5$	—	—	25	7.31_2



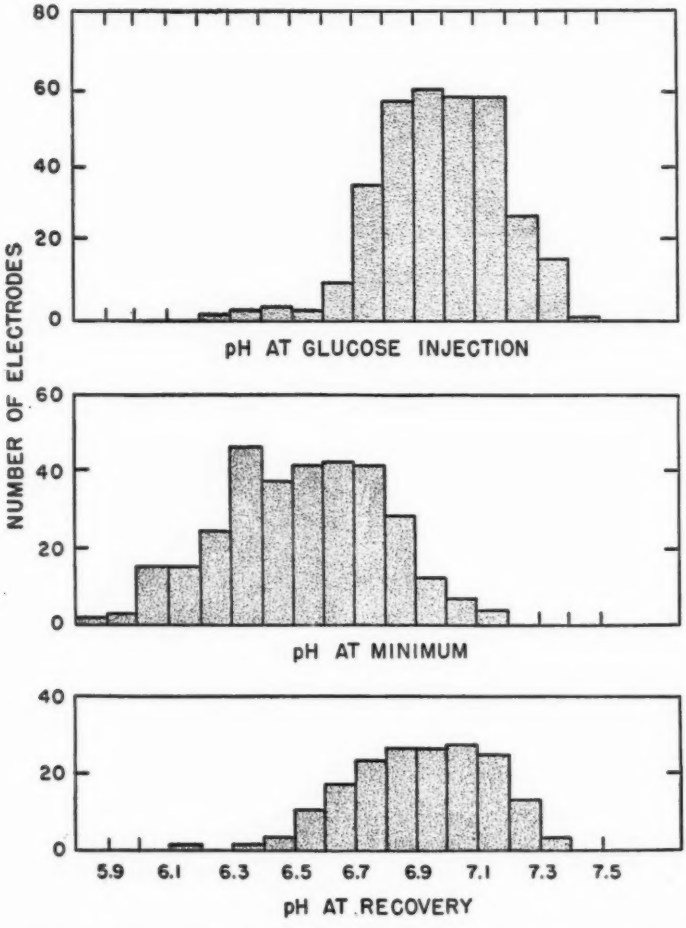
TEXT-FIGURE 1.—Recorded chart showing *p*H *versus* time in hours of 6 glass electrodes at different sites within a single 3741 tumor.



TEXT-FIGURE 2.—*p*H response to injection of glucose at 2 hours. Data on rat tumors are the averages of figures computed from 338 electrodes, on normal muscle, from 29 electrodes.

made during the early course of these researches were incomplete. In a number of experiments the rats failed to show any signs of recovery; consequently no data were available for computing the time to minimum *p*H, the recovery rate, and the *p*H at complete recovery. The quantities were computed only from the data on those electrodes which indicated a complete *p*H recovery of the rat tissue. We considered recovery to be complete when the rate of increase of *p*H was less than 0.02 *p*H unit per hour. The value of *p*H at the end of the run was not used as a measure of the completeness of recovery.

Inspection of text-figure 1 shows wide variation in the recorded potentials (*p*H) from several electrodes inserted at different locations within a single tumor. This raises the question of the relative magnitudes of deviation within and between tumors. To answer this question the data on 23 lymphosarcomas, in each of which 2 glass electrodes were inserted, were analyzed by the method described by Youden (*27*). The variance, or standard deviations squared, within tumors was 0.0111 and between tumors was 0.0225; the total variance 0.0336 equalling the sum of the 2 subvariances. The standard deviation within tumors was 0.105 and be-



TEXT-FIGURE 3.—Frequency diagrams for all tumors studied, showing number of measurements in each *p*H range of 0.1 unit. *p*H distribution before glucose injection—*top diagram;* distribution of *p*H at the minimum of the *p*H chart after glucose injection—*middle diagram;* *p*H recovery near the termination of the run—*bottom diagram.*

tween tumors 0.150, the total standard deviation being 0.183. The F test indicates that the total *p*H difference between tumors is significantly higher than within tumors, but nevertheless the variation in *p*H within tumors is sufficiently high to warrant further investigation.

Sixteen rats died during the course of the experiment and were not included in the tabulation. In addition, there were occasions when instrumental disturbances rendered portions of a record undecipherable.

The data are summarized in another form in text-figures 3 and 4. The ordinate in the bar graphs represents the number of electrodes at each interval of the abscissa. The data for all the rats are included in the graphs of text-figures 3 and 4.

Since the most complete series of results was obtained with rats bearing the Murphy-Sturm lymphosarcoma, these data have been summarized separately in text-figures 5 and 6. This tumor was the only one of the series studied which remained free of necrotic tissue.

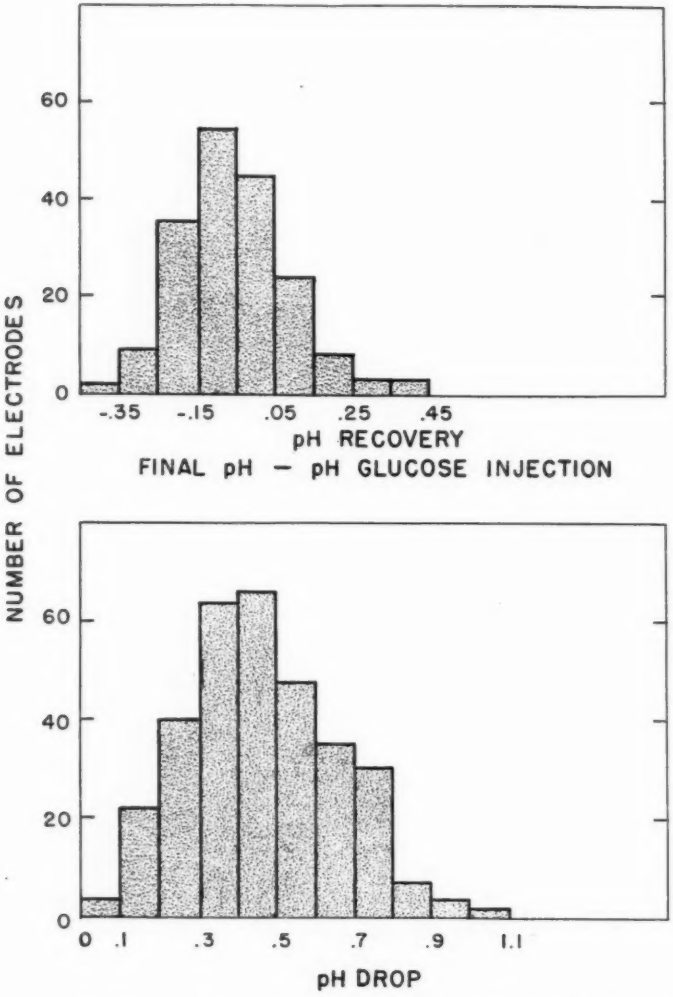
A series of experiments was performed in which a second injection of glucose was administered some time after the first injection. The results of these experiments are given in table 3.

Since the temperature of the rats usually dropped during the course of the experiments, it was considered worthwhile to investigate the effect of temperature on the *p*H response. The temperature response was measured thermoelectrically in a few rats. Fine copper-constantan thermocouples were inserted *per anum* or else into a small hole in the muscle or tumor sheath and forced into the tissue. These data were recorded on the strip chart of a 16-point potentiometer recorder with a sensitivity of 0.2° per scale division (*ca.* 1 mm.). The results of these preliminary experiments may be summarized by the statement that no definite corre-

TABLE 3.—p*H response to a second glucose injection*

Run number	Number of rats	Number of electrodes	Initial *p*H (*average*)	Minimum *p*H (*average*)	Time to second glucose injection	*p*H at second glucose injection (*average*)	Second minimum *p*H (*average*)	Remarks
1	2	6	7. 04	6. 65	20°30′	7. 08	6. 89	Completely recovered prior to second glucose injection.
2	3	6	6. 75	6. 41	23°30′	6. 91	6. 53	Completely recovered prior to second glucose injection.
3	3	6	6. 89	6. 46	15°15′	6. 68	6. 30	Incomplete recovery at time of second glucose injection.
4	3	6	6. 97	6. 64	3°15′	6. 84	6. 57*	Incomplete recovery at time of second glucose injection.

*20 hours later *p*H had recovered to 7.03.

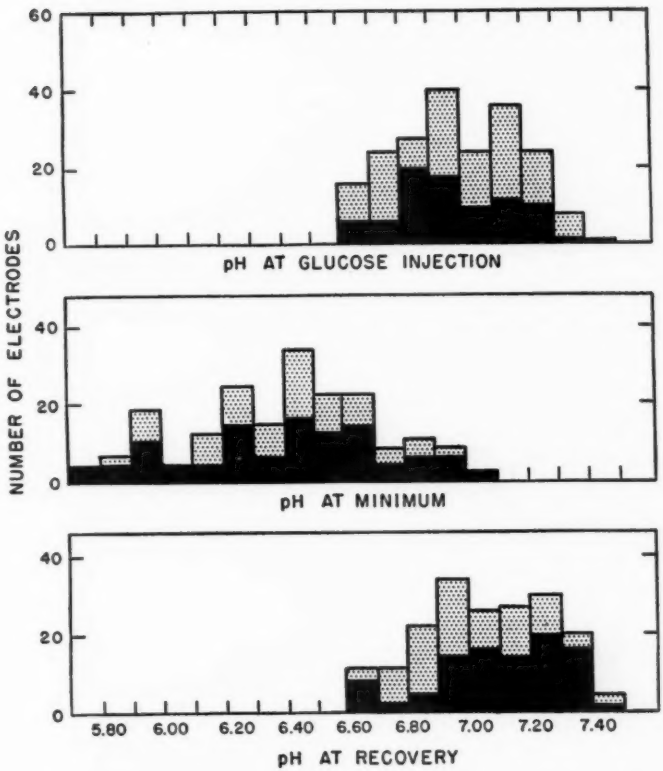


TEXT-FIGURE 4.—Frequency distribution of the final *p*H minus the *p*H at glucose injection for all tumors—*top diagram;* drop in *p*H between initial value and value at *p*H minimum after glucose (all tumors)—*bottom diagram.*

lation was found between temperature and either initial or minimum *p*H of the rats.

Discussion of Results

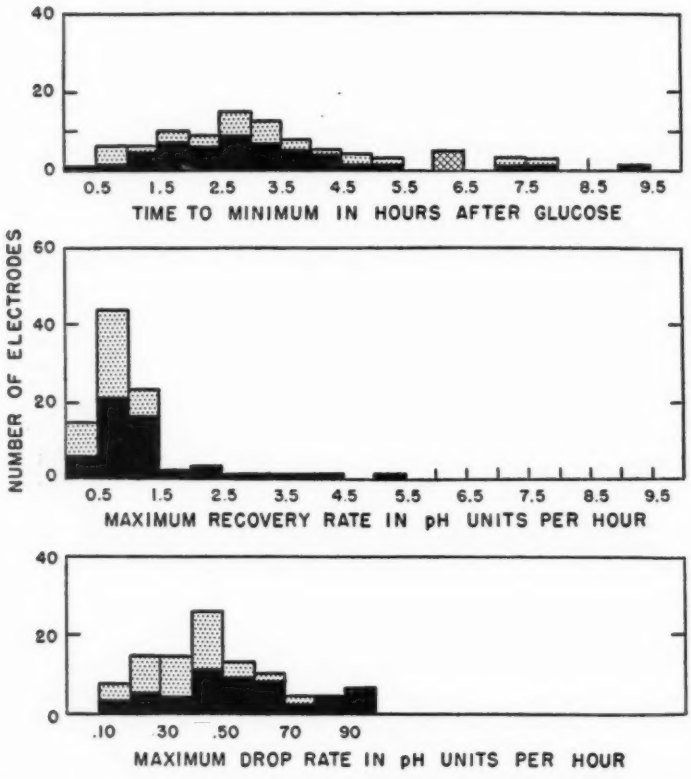
Any discussion of the results of an *in vivo* experiment, such as this one, must be tempered by an awareness of the numerous physiological perturbations that such an experiment induces. The test animals are, in some measure, debilitated by the tumors they bear. We must assume



TEXT-FIGURE 5.—Frequency distribution for *p*H at glucose injection—*top diagram;* *p*H minimum after glucose—*middle diagram;* at recovery—*bottom diagram.* Lymphosarcoma in Sprague-Dawley rats—*solid areas;* in Wistar rats—*dotted areas.*

that they suffer a certain trauma from the anesthetic, from the injection of sugars, from the restraining bindings, and from the insertion of the electrodes. The restraining bindings, the anesthetic, and glucose injection all result in a lowering of the body temperature and a decrease in respiratory rate and blood flow. The insertion of the electrode into living tissue is usually followed by a *p*H shift which is largely overcome in the course of an hour's time, when a "steady" value is attained. The electrode system measures the *p*H of a pocket of extracellular fluid derived from the cellular debris produced by the insertion of the electrode and from the fluid exchange of adjacent cells.

The magnitude of these disturbing effects must be evaluated before conclusions may be drawn concerning the *p*H change caused by glycolysis. Previous work (*3*) had shown that galactose and lactose with molecular weights of 180 and 360, respectively, (the former having approximately the same osmotic pressure as glucose, molecular weight 180) produced a *p*H change in Jensen sarcomas and Flexner-Jobling carcinomas of less than



TEXT-FIGURE 6.—Frequency distribution for time to minimum in hours after glucose—*top diagram;* maximum recovery in *p*H units per hour—*middle diagram;* maximum drop rate in *p*H units per hour—*bottom diagram.* Lymphosarcoma in Sprague-Dawley rats—*solid areas;* in Wistar rats—*dotted areas.*

0.1 *p*H unit. Additional controls, using lactose on rats bearing lymphosarcomas and galactose on rats bearing tumors 1643 and 3924, indicated a total *p*H drop of 0.08 and 0.06, respectively. This indicates that the cumulative effects of all the physiological disturbances is less than 0.1 *p*H unit. The fact that certain sugars have little effect upon the *p*H response indicates that the stasis in capillaries and decreased vascularity in tumors described by Algire and Legallais (*28*) following massive glucose administration is not the primary cause of the *p*H change. This conclusion was confirmed by injecting anesthetized rats which carried the lymphosarcoma with podophyllotoxin (in doses of 7.5 mg. per kg.), which reduces the blood circulation in tumors (*29*). No change whatever was seen in the *p*H response curve.

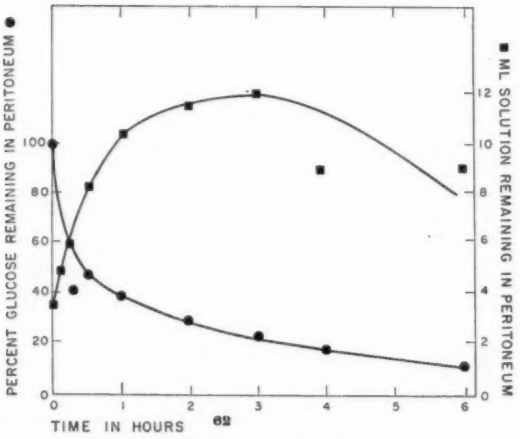
In the course of an experiment of long duration, the outpouring of fluids from the tissues into the peritoneum following the injection of the glucose must lead to an extensive dehydration of the animal. The

observation that most of the electrodes return to a *p*H value close to the same as that at the beginning of each experiment indicates that the mechanics of *p*H response and recovery are essentially independent of dehydration effects, since these effects are not reversed during the course of the experiment. Kahler and Buchanan (*30*) observed that fluid begins to flow into the peritoneum immediately after massive injection of glucose. The extracellular fluid draining into the peritoneum reaches a maximum of approximately 12 ml. about 3 hours after the injection. We have determined that the amount of fluid remaining in the peritoneal cavity after 22 hours is negligible; so we may assume, then, that the water that was injected as glucose solution (3 ml. per 100 gm. of body weight) will go toward decreasing the water deficit. However, the normal water turnover in rats is 0.60 to 0.75 gm. per 100 gm. of body weight per hour, most of which is accounted for by loss through evaporation (*31*). Even if we assume the lower value, the rats in our experiments lost about 14 gm. of water per 100 gm. of body weight in a 24-hour period, so that they were considerably dehydrated at the end of the experimental run in spite of the fluid injected.

Even though it is reasonable to suppose there is some delay between the injection of glucose and the *p*H response, we have not been able to observe it in the case of most lymphosarcomas. The technique employed in these experiments is such that 2 to 10 minutes may elapse from the time of injection to the first recorded point. In our experiments the *p*H usually dropped at this first postinjection determination and, in fact, the initial portion of the curve showed the steepest descent. The rate of fall decreased with time, until a minimum was reached in the *p*H curve. After the *p*H began to rise, the ascending portion of the curve passed through a point of inflection, and ultimately became asymptotic to a *p*H value not very different from the initial *p*H. It appears that the "lag time" or "latent period" is probably shorter than 2 minutes for most tumors. However in some cases, tumors, such as 1643, have a "lag time" up to 20 minutes.

Since it is reasonable to assume that the *p*H drop observed in the extracellular fluid of tumor tissue is associated with the absorption and metabolism of glucose in the adjacent cells, it is of interest to compare this response with the rate of transfer of glucose from the peritoneal cavity into the tissues. For this purpose we have plotted in text-figure 7 the results computed from the data of Kahler and Buchanan (*30*). These data were obtained by injecting glucose doses similar to the ones we have used and then measuring both the amount of water and the concentration of glucose remaining in the peritoneum as a function of time. Within 6 minutes 300 mg. of glucose per 100 gm. of body weight, which was about half the initial dose, had passed from the peritoneal cavity into the blood stream. Thus, the passage of glucose from the peritoneum to the blood stream and thence to tissue may be sufficiently rapid to explain the speed with which the *p*H of the tumor extracellular fluid begins to fall.

Our results also suggest that one injection of glucose does not alter the



TEXT-FIGURE 7.—Number of milliliters of solution in peritoneum *versus* elapsed time in hours after injection of 3.7 ml. of a 19.1 percent glucose solution per 124 grams of rat weight—*squares;* percent of total glucose injected remaining in peritoneum after time in hours—*circles.* Data of Kahler and Buchanan (*30*).

response of the test animal following a second injection of glucose. The qualitative results of table 3 have been extended in a series of determinations that will be described in a forthcoming publication.

It had occurred to us that there might be some correlation between the initial *p*H and the extent of the *p*H drop. Thus, it might be conjectured that tumor tissues exhibiting initial *p*H values close to that of normal tissue would behave more nearly like normal tissue and present a smaller *p*H drop. Or it might be conjectured that if the initial *p*H were low the buffering capacity of the tissue would prevent it from falling much lower. However, no interpretation can be made for the direct plot of *p*H drop as a function of initial *p*H because the scatter of values is too great. The tabulation of initial tumor *p*H *versus* *p*H drop is presented in two ways in table 4. These results indicate that the *p*H drop following glucose

TABLE 4.—*Relation between initial* p*H and extent of* p*H drop*

Initial *p*H as independent variable			Δ *p*H as independent variable		
*p*H interval	Number of electrodes	Δ *p*H average	Δ *p*H interval	Number of electrodes	Initial *p*H average
6. 30–6. 39	3	$.22_2$	0– . 09	3	7. 07
6. 40–6. 49	3	$.27_0$	. 10– . 19	29	$6. 95_5$
6. 50–6. 59	4	$.29_5$	. 20– . 29	40	$6. 96_7$
6. 60–6. 69	6	$.53_2$	. 30– . 39	64	$7. 01_0$
6. 70–6. 79	33	$.48_3$	. 40– . 49	65	$6. 98_9$
6. 80–6. 89	52	$.44_4$	. 50– . 59	53	$6. 95_8$
6. 90–6. 99	62	$.43_2$	. 60– . 69	32	$7. 01_6$
7. 00–7. 09	61	$.43_3$	. 70– . 79	27	$6. 98_5$
7. 10–7. 19	57	$.45_9$	. 80– . 89	8	$6. 92_9$
7. 20–7. 29	29	$.41_6$	. 90– . 99	1	7. 35
7. 30–7. 39	16	$.42_9$	1. 00–1. 09	1	7. 11

injection is approximately the same over a range of initial tumor *p*H values varying from 6.7 to 7.3.

Summary

Previous work on the *p*H of rat tumors measured with the capillary glass electrode has been extended to 8 more tumor types, measurements having been made on 338 electrodes. The average initial *p*H of the tumors was 6.99. After injection of a massive dose of glucose the *p*H dropped to 6.55 in a time interval which was usually between 3 and 4 hours. After this the *p*H slowly returned to a value approximating the initial value.

Normal rat leg muscle (29 electrodes) gave an initial value of 7.396 and, after glucose injection, gave a value of 7.258.

The standard deviation of 23 pairs of electrodes in 23 lymphosarcomas was 0.105 *p*H unit within tumors while between these tumors it was 0.150, the total deviation being 0.183. In the case of tumor 3741, the standard deviation for multiple electrodes within a single tumor was 0.123.

The *p*H differences between tumors as well as within tumors was always greater following glucose administration. On the other hand, normal muscle had about the same standard deviation following glucose as it had initially.

It was concluded that the rapid fall in tumor *p*H immediately following intraperitoneal glucose administration could be correlated with the rapid assimilation of glucose from the peritoneum.

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The Nutrition of Animal Tissues Cultivated *In Vitro*. I. A Survey of Natural Materials as Supplements to Synthetic Medium 199[1]

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The development of a chemically defined medium, Mixture 199 (*1*), has made it possible to cultivate certain freshly explanted tissues for prolonged periods of time under precisely controlled conditions. This medium has found wide application in studies on virus propagation in tissue culture (*2–4*) and in studies on tissue cell metabolism (*5*). However, tissues cultivated in medium 199 do not exhibit the extensive proliferation and prolonged survival characteristic of tissues cultivated in natural media containing serum and chick-embryo extract. Since these complex natural materials must, therefore, contain unidentified substances essential for cell proliferation and survival, it was considered of interest to employ medium 199 as a base and to add to it graded levels of a wide variety of natural materials of known biological activity. From the effect of such substances in either increasing cell proliferation or prolonging cell survival it was hoped to obtain a distribution pattern of activity that might indicate the chemical nature of the active ingredients.

Materials and Methods

Synthetic Media

During the 5 years since the first studies on medium 199 (*1*), considerable improvements and simplifications in its preparation have been made. These include the substitution of Hanks' balanced salt solution (*6*) for Earle's solution (*7*), providing a new basal medium designated as M 150. These modifications have been tested on thousands of cultures and have been in routine use in this laboratory for the past 2½ years. Extensive experiments have shown that M 150 maintained cell survival for the same 35- to 45-day period characteristic of 199 and also supported equal propagation of influenza and mumps viruses in cultures of chorioallantoic membrane (*4*). It is interesting to note that the preparation of 199 in Hanks' solution has recently been recommended by other virus workers (*8*). In view of the widespread use of medium 199, it seems of value to report these modifications in detail at the present time.

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It has been found most convenient to prepare a series of 14 stock solutions, each with the following composition:

Solution 1.—L-Arginine HCl, 140 mg.; L-histidine HCl, 40 mg.; L-lysine HCl, 140 mg.; DL-tryptophane, 40 mg.; DL-phenylalanine, 100 mg.; DL-methionine, 60 mg.; DL-serine, 100 mg.; DL-threonine, 120 mg.; DL-leucine, 240 mg.; DL-isoleucine, 80 mg.; DL-valine, 100 mg.; DL-glutamic acid monohydrate, 300 mg.; DL-aspartic acid, 120 mg.; DL-α-alanine, 100 mg.; L-proline, 80 mg.; L-hydroxyproline, 20 mg.; glycine, 100 mg.; sodium acetate trihydrate, 166 mg.; phenol red (water soluble), 40 mg. These ingredients are dissolved by thorough mixing in 1,000 ml. of double-strength Hanks' balanced salt solution (*6*) containing neither glucose nor sodium bicarbonate.

Solution 2.—L-Tyrosine, 200 mg.; L-cystine, 100 mg. These materials are dissolved with heat in 100 ml. of 0.075 *N* HCl.

Solution 3.—B vitamin group. Niacin, 25 mg.; niacinamide, 25 mg.; pyridoxine HCl, 25 mg.; pyridoxal HCl, 25 mg.; thiamine HCl, 10 mg.; riboflavine, 10 mg.; calcium pantothenate, 10 mg.; *i*-inositol, 50 mg.; *p*-aminobenzoic acid, 50 mg.; choline chloride, 500 mg.; ion-exchange water to 200 ml.

Solution 4.—Fat-soluble group. Prepare unsterile solutions in absolute or 95 percent ethyl alcohol of cholesterol (10 mg. per ml.) and menadione [vitamin K (10 mg. per ml.)]. Weigh out 10 mg. of calciferol (vitamin D_2) and dissolve in 2 ml. of the alcoholic cholesterol solution. Then add 0.1 ml. of alcoholic vitamin K solution. Use this combined alcoholic solution to dissolve 10 mg. of vitamin A. Then add 10 ml. of 5 percent aqueous Tween 80 solution and dilute the turbid mixture to 100 ml. with ion-exchange water. This solution remains opalescent but becomes clear when incorporated in the final medium.

Solution 5.—Vitamin C mixture. L-Cysteine HCl, 100 mg.; glutathione, 50 mg.; ascorbic acid, 50 mg.; ion-exchange water to 100 ml.

Solution 6.—Disodium α-tocopherol phosphoric acid, 10 mg.; ion-exchange water to 100 ml.

Solution 7.—Folic acid, 10 mg.; ion-exchange water to 100 ml. with the inclusion of just sufficient *N*/100 NaOH to effect solution.

Solution 8.—*d*-Biotin, 10 mg.; 1.0 ml. of 1.0 *N* HCl; ion-exchange water to 100 ml.

Solution 9.—Adenine sulfate, 100 mg.; dissolve with heat in 100 ml. of ion-exchange water containing 0.5 to 0.7 ml. of concentrated NH_4OH.

Solution 10.—Purine and pyrimidine mixture. Guanine HCl, 10 mg.; monosodium xanthine, 11.4 mg.; hypoxanthine, 10 mg.; thymine, 10 mg.; uracil, 10 mg.; dissolve with heat in 200 ml. of ion-exchange water containing 0.5 ml. of concentrated NH_4OH.

Solution 11.—Adenosinetriphosphate, disodium, 27 mg.; ion-exchange water to 50 ml. Prepare fresh each week.

Solution 12.—Iron solution. Dissolve 36.0 mg. $Fe(NO_3)_3 \cdot 9H_2O$ in 100 ml. of ion-exchange water containing 1 drop of concentrated HNO_3.

Solution 13.—Muscle adenylic acid, 10 mg.; ion-exchange water to 100 ml.

Solution 14.—*d*-Ribose, 100 mg.; d-2-deoxyribose, 100 mg.; ion-exchange water to 100 ml.

These 14 stock solutions and, in certain cases, the working solutions prepared from them are kept in the refrigerator under nonsterile conditions. However, solutions 2, 9, and 10, which are rather insoluble, are sterilized by filtration and stored at room temperature. To prepare the final medium, appropriate volumes of the various solutions are combined, as listed in table 1. In this final medium are incorporated 1,000 mg. per liter of glucose, 100 mg. per liter of L-glutamine, and 1.4 gm. per liter of sodium bicarbonate. Antibiotics are added as required, the final volume is adjusted with ion-exchange water, and the medium is sterilized by passage through fritted glass filters.

TABLE 1.—*Method of preparation of synthetic medium M 150**

Solution No.	Composition	Dilution of stock solution †	Ml. per 1,000 ml. of final medium
1	Amino acids in 2 × Hanks'	Undil.	500
2	Tyrosine and cystine‡	Undil.	20
3	B vitamin group	1/50	10
4	Fat-soluble group	1/10	10
5	Vitamin C mixture	1/100	10
6	Vitamin E	1/100	10
7	Folic acid	1/100	10
8	Biotin	1/100	10
9	Adenine sulfate‡	Undil.	10
10	Purines and pyrimidines‡	Undil.	6
11	Adenosinetriphosphate	Undil.	2
12	Iron solution	Undil.	2
13	Adenylic acid	Undil.	2
14	Ribose and deoxyribose	1/4	2
	Ion-exchange water to		1,000 ml.
	Sodium bicarbonate		1,400 mg.
	Glucose		1,000 mg.
	Glutamine		100 mg.

*Antibiotics may be added to the final medium at the required concentration.
†Ion-exchange water is employed for all stock and working solutions (*9*).
‡Solutions kept at room temperature after sterilization.

The revised method of preparing medium M 150 (table 1) embodies a number of changes from the original preparation of medium 199 as previously published (*1*). To avoid confusion, these changes may be listed as follows:

(a) The original formula for 199 specified that the organic ingredients be dissolved in Earle's salt solution (*7*). During the long-term studies reported in the present communication, however, it was observed that toxic precipitates tended to form and that this could be avoided if the medium were prepared in Hanks' balanced salt solution (*6*). The reduced levels of calcium and bicarbonate appeared responsible for the greater stability. Moreover, media prepared in Hanks' solution possessed a *p*H of about 7.2, and cultures in this medium did not require gassing with mixtures rich in carbon dioxide.

(b) The fat-soluble compounds, originally kept as separate stock solutions, have been combined into a single solution.

(c) Folic acid has been dissolved in water, with the aid of dilute sodium hydroxide.

(d) Xanthine has been replaced in the purine and pyrimidine mixture by the more soluble monosodium xanthine.

(e) The barium salt of adenosinetriphosphate has been replaced by the water-soluble, neutral sodium salt.

(f) Adenylic acid, ribose, and deoxyribose are kept as separate solutions and are not incorporated in the stock amino acid mixture.

(g) Sodium bicarbonate, glucose, and glutamine are not included in the stock amino acid mixture, but are weighed out as separate ingredients and dissolved in the final medium shortly before filtration. This step reduces the loss of labile amide nitrogen from glutamine and minimizes the possible interactions between glucose and amino acids under mildly alkaline conditions.

(h) Antibiotics have been included in the medium. For nutrition experiments, sodium penicillin G at a level of 10 units per ml. was found sufficient to control chance bacterial contamination. For virus studies, higher levels of both penicillin and streptomycin are recommended (*4*).

With the exception of chick-embryo extract, which was prepared as described previously (*12*) but with the substitution of Hanks' for Earle's balanced salt solution, the natural materials tested were obtained commercially. Yeast extract, beef extract, and horse serum were obtained from Difco Laboratories, Detroit; whole-liver extract from Sigma Chemical Company, St. Louis; liver fraction L from Nutritional Biochemicals, Inc., Cleveland; amniotic fluid from Cudahy Laboratories, Omaha; liver coenzyme concentrate from Armour and Co., Chicago; and ascitic fluid from Microbiological Associates, Inc., Bethesda. Experimental media were prepared by dissolving graded levels of the test materials in the M 150 base and adjusting the *p*H to approximately 7.2 when required. All media were prepared at frequent intervals and sterilized by passage through UF fritted glass filters.

Tissue Cultures

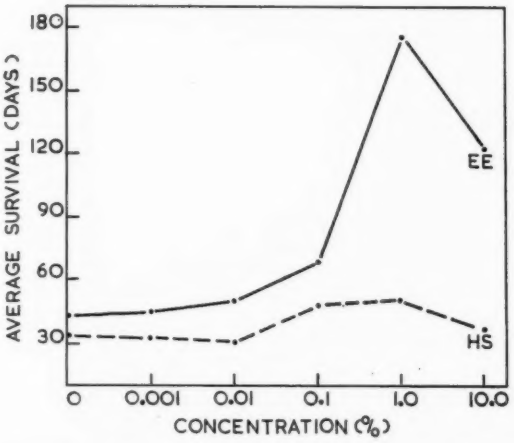
All cultures were prepared from the heart muscle of 11-day-old chick embryos. The tissues were chopped to a fine pulp and portions transferred to standard pyrex test tubes by a capillary pipette. Cultures prepared in this way contained from 1 to 2 mg. of tissue, wet weight, as determined by frequent weighings of replicate samples. After a 25- to 30-minute period during which the tissue became fixed directly to the glass without the use of plasma clots, medium M 150 was added and the cultures incubated at 37° C. by the conventional roller-tube technique (*10*). Full details of these culture procedures have been reported previously (*1, 5, 11*).

Each experiment employed 80 cultures, prepared at one time from a

pooled lot of tissue. During the initial 7-day period, all cultures were grown in 0.75 ml. of M 150. At the end of this time, the cultures were examined microscopically and divided into equivalent groups of 10 to 15 cultures each. One group was kept as a control in M 150 and the starting medium was then removed and replaced in each tube by 1.5 ml. of experimental or control medium. All media were completely renewed once each week and cultures were examined microscopically at frequent intervals until living cells could no longer be seen. All experiments were carried out in duplicate and results were calculated as the average survival times of the groups of cultures in each test medium. The significance of differences in survival times was calculated by the standard t test. Although this method is highly subjective, statistical analysis of the data has shown that small differences in survival times can be very significant (*5*).

Results

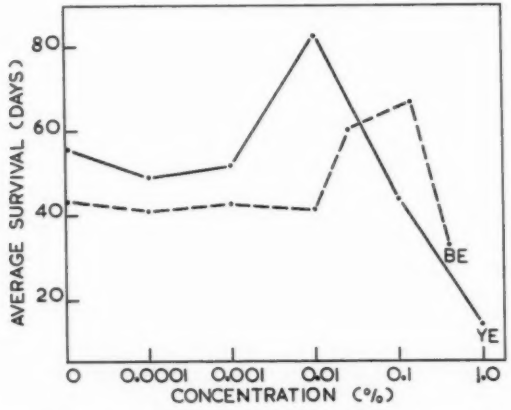
Effect of graded levels of chick-embryo extract and horse serum.—Media containing embryo extract and serum are known to produce extremely active short-term migration and proliferation with chick heart tissue, and, consequently, these materials were the first ones tested as supplements in medium M 150, with the results shown in text-figure 1. It is apparent that chick-embryo extract had a marked effect on culture survival, with the maximal response occurring at 1 percent (expressed in terms of the original volume of embryonic tissue pulp used in preparing the extract). At concentrations above and below this level, the effect on culture survival was much less. With horse serum, increased culture survival was obtained only at concentrations of 0.1 and 1.0 percent, and the observed increase was relatively much less than in the case of chick-embryo extract.



TEXT-FIGURE 1.—Effect of graded levels of horse serum (HS) and chick-embryo extract (EE) on tissue-culture survival.

Effect of graded levels of yeast extract and beef extract.—Yeast and beef extracts are commonly used to supply growth-promoting substances in bacteriological media, and, therefore, these materials were next assayed for their effects on tissue cultures, with the results shown in text-figure 2. It is evident that both these materials caused a marked increase in tissue cell survival and that concentrations greater than the stimulating levels caused a high degree of toxicity.

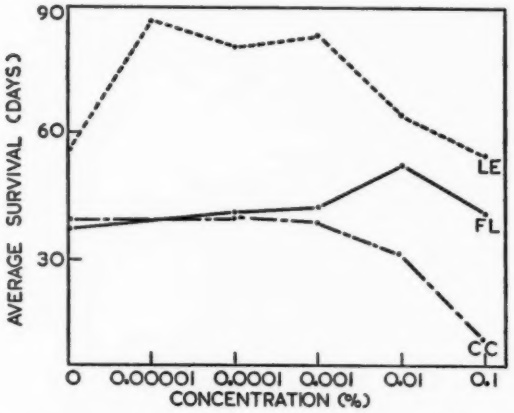
Effect of graded levels of liver fractions.—Since liver is commonly regarded as a good source of unidentified growth factors, various liver fractions were investigated in considerable detail. The effect on tissue cell survival exerted by graded levels of whole-liver extract, liver fraction L, and liver



TEXT-FIGURE 2.—Effect of graded levels of yeast extract (YE) and beef extract (BE) on tissue cell survival.

coenzyme concentrate is shown in text-figure 3. Whole liver extract exerts a pronounced effect in prolonging cell survival, even at extremely low concentrations. Liver fraction L caused a moderate increase in survival, but only at high concentrations. Liver coenzyme concentrate caused no increase in survival of the cultures at any level, while toxicity increased with concentration. Since these experiments were carried out over a 2-year period, considerable variation in the control values with M 150 was obtained, due, presumably, to seasonal variation in the fertile eggs. However, at any season the activity of the material under test relative to the control value remained constant. Thus, the value of 56 days, recorded for the controls on whole-liver extract, was somewhat high, but the beneficial effect of this material was reproducible in repeated experiments including concentrations higher and lower than those shown on the graph.

Effect of graded levels of bovine amniotic fluid, ascitic fluid, and corn-steep liquor.—No major effect was obtained with any of these three compounds (text-fig. 4). Bovine amniotic fluid caused a slight increase in cell survival at a concentration of 1.0 percent and a similar slight increase was obtained

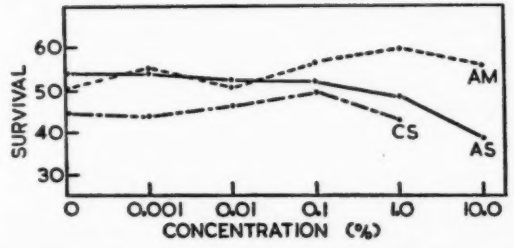


TEXT-FIGURE 3.—Effect of graded levels of whole-liver extract (LE), liver fraction L (FL), and liver coenzyme concentrate (CC) on tissue cell survival.

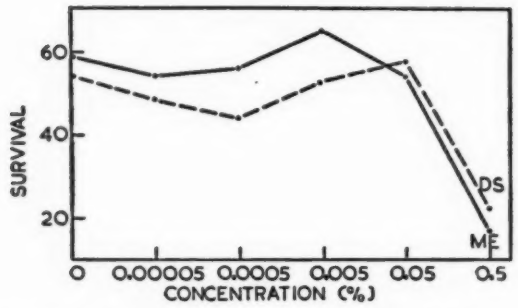
with corn-steep liquor, but ascitic fluid showed only a gradual toxicity with increasing concentration.

Effect of graded levels of malt extract and distillers' dried solubles.—The effect of these two substances on tissue cell survival is shown in text-figure 5. It is evident that no significant increase in survival has been exerted but, rather, a sharp toxicity with increasing concentration in both cases.

Relative activity of natural materials tested.—From the data obtained in this survey, the relative activity of the various natural materials tested may be determined (table 2). When these substances are listed in order of decreasing activity, based on the percentage increase in cell survival (columns 1 and 2), embryo extract is found to be by far the most active substance, followed by whole-liver extract, beef extract, horse serum, yeast extract, and liver fraction L, respectively. The other compounds tested, with the exception of bovine amniotic fluid, showed little or no survival-promoting activity. However, when the order of relative activity is based on the concentration necessary for maximal stimulation (columns 3 and 4), an entirely different sequence is obtained. The most active substance now appears to be whole-liver extract, followed by yeast



TEXT-FIGURE 4.—Effect of graded levels of amniotic fluid (AM), ascitic fluid (AS) and corn-steep liquor (CS) on tissue cell survival.



TEXT-FIGURE 5.—Effect of graded levels of malt extract (ME) and distillers' dried solubles (DS) on tissue cell survival.

extract, liver fraction L, and beef extract. Embryo extract, which ranked first by the previous calculation, ranks only fifth by this method of interpretation. It would appear, then, that in studying the survival-promoting activity of natural materials, both the magnitude of the effect and the relative effective concentration must be considered.

TABLE 2.—*Relative survival-promoting activity of natural materials tested*

Substance tested*	Increase in survival time (*percent*)	Substance tested*	Concentration for maximal stimulation (*percent*)
Chick-embryo extract	312.0	Whole-liver extract	0.00001
Whole-liver extract	55.5	Yeast extract	.01
Beef extract	55.0	Liver fraction L	.01
Horse serum	50.0	Beef extract	.16
Yeast extract	48.0	Embryo extract	1.0
Liver fraction L	41.5	Horse serum	1.0
Amniotic fluid†	19.0	Amniotic fluid	1.0
Corn-steep liquor	10.0‡	Corn-steep liquor	—
Malt extract	8.5	Malt extract	—
Distillers' solubles§	6.5	Distillers' solubles	—
Liver coenzyme	—	Liver coenzyme	—
Ascitic fluid‖	—	Ascitic fluid	—

*The source of materials tested is listed in the text under Materials and Methods.

†Obtained through the courtesy of Dr. H. J. Anderson, Cudahy Laboratories, Omaha.

‡Increases in survival times of less than 15 percent were not found to be statistically significant by the *t* test.

§Obtained through the courtesy of Dr. W. L. Holmes, Department of Pharmacology, Yale University, New Haven.

‖Obtained through the courtesy of Mr. M. M. Vincent, Microbiological Associates, Inc., Bethesda.

Discussion

The results of the present survey, which are based on more than 3,000 cultures over a 2-year period, have shown the wide distribution in natural materials of substances promoting the survival of tissue cultures. Although earlier workers (*13*) had demonstrated the beneficial effect of certain natural materials [for review see (*14*)], the present survey is the first one made on a comparative basis, employing a known synthetic

medium base. Under these circumstances, it was of particular interest to determine the effect of horse serum and chick-embryo extract, which are constituents of the usual nonsynthetic culture medium. The results obtained (text-fig. 1) show that horse serum does prolong culture survival but not to a very great extent. With chick-embryo extract, however, the increase in cell survival is very pronounced, since many cultures lived 8 to 10 months. These results suggest that medium M 150 serves as a reasonably complete substitute for horse serum but not for chick-embryo extract. It is of interest to note that fractionation attempts with chick-embryo extract have not yet been successful in either concentrating or purifying the active ingredients (*15*). The components of embryo extract so far identified do not account for the unusual properties of this material (*16*).

Studies with various commercial liver preparations (text-fig. 3) show that whole liver is a very potent source of survival-promoting substances for freshly explanted chick embryonic-heart tissue, since marked increases in cell survival were obtained at liver concentrations as low as 0.00001 percent. Fractionation of liver extract, as done commercially, results in a rapid loss of activity, since liver fraction L possesses only approximately one-thousandth the activity of the whole extract as judged by the maximal effective concentration, and liver coenzyme concentrate is devoid of activity. From these results, it would appear that tissue cells have no requirement for strepogenin (*17*). Moreover, the activity of liver extract cannot be attributed to vitamin B_{12}, since experiments with this material (unpublished) have shown only a minor response. It is of interest that liver coenzyme concentrate has recently been reported to markedly increase cell proliferation in cultures of Earle's L strain, clone 929 (*18*) maintained in a modified medium 199 (*19*). Under the conditions of the present experiments, in which fresh chick embryonic-heart fibroblasts were used, the same material at a wide range of concentrations had no effect on cell survival other than a toxicity that increased considerably with concentration. These divergent results suggest that, in studies on the nutrition of tissue cultures, a clear distinction must be made between cell proliferation and cell survival. It appears probable that synthetic media devised for short-term cell proliferation will differ, both in composition and concentration, from synthetic media devised for long-term culture maintenance and survival.

The distribution pattern of activity in natural materials did not provide a clear distinction between substances of animal and of plant origin. With certain important exceptions, however, the majority of the animal materials tested were active and the majority of the plant materials were inactive (table 2). The lack of activity in malt extract in these experiments is not in agreement with the findings of earlier workers (*20*), who employed a dialyzed medium base. The observation that distillers' dried solubles had no beneficial effect suggests that, under these experimental conditions, tissue cultures have no requirement for vitamin B_{14} (*21*).

The purpose of the present survey was to attempt to obtain information on the nature of the unidentified factors required for tissue cell nutrition through the distribution pattern of activity in natural materials. The general observation (table 2) that greatest activity is found in liver, yeast, and embryo extract suggests that the active ingredients might be coenzymic in nature, and recent experiments (unpublished) have shown that coenzyme A will duplicate some of the responses obtained with natural materials. However, the finding that liver coenzyme concentrate, which is rich in coenzyme A, is toxic rather than beneficial suggests that the active substances may exist in nature in close association with toxic materials. It is hoped that this survey may provide a useful basis for future investigation of this relationship.

Summary

1) Recent modifications and improvements in the preparation of synthetic medium M 150 (modified Mixture 199) have been described.

2) Employing M 150 as a basal maintenance solution, a survey has been made of the distribution in natural materials of survival-promoting substances for freshly explanted chick embryonic-heart tissues cultivated in roller tubes without plasma clots.

3) When relative increase in cell survival was employed as the criterion, greatest activity was found in chick-embryo extract, followed, in order of decreasing activity, by whole-liver extract, beef extract, horse serum, yeast extract, liver fraction L, and bovine amniotic fluid.

4) When effective stimulating concentrations served as the criterion, greatest activity was found in whole-liver extract, followed, in order of decreasing activity, by yeast extract, liver fraction L, beef extract, chick-embryo extract, horse serum, and bovine amniotic fluid.

5) No stimulating activity was found with corn-steep liquor, malt extract, distillers' dried solubles, liver coenzyme concentrate, or ascitic fluid.

6) The implications of these findings to cell nutrition studies are discussed.

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Desmoid Tumor of the Popliteal Space Occurring During Pregnancy: Report of a Case with Bioassays[1]

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In their study of the abdominal wall as a surgical organ, Pack and Ehrlich (*1*) define desmoid tumors as unencapsulated, infiltrating fibromas of fascial or aponeurotic origin. They observe that desmoid tumors in this location are more common in women than in men and that all but one of their female patients had recently been pregnant, the tumor having often been first observed during pregnancy. They discuss the possible etiologic background of endocrine factors as well as the more conventional theories regarding the role of an unhealed injury (*e.g.*, stretching of the fascial or aponeurotic fibers during pregnancy). In a report on extra-abdominal desmoid tumors Musgrove and McDonald (*2*) found that of 34 cases 9 were situated in the lower extremity and only 3 in or near the popliteal space. Calculation from data in this report indicates that the occurrence of desmoid tumors is twice as common in the anterior abdominal wall as in extra-abdominal locations.

There is both clinical and experimental evidence that desmoid tumors are in some way related to hormonal activity. Most frequently emphasized is their tendency to occur in young women during or shortly after gestation. Less often cited is the observation by Ewing (*3*) that these fibrous tumors respond slowly but satisfactorily to irradiation, an effect one would hesitate to predict on *a priori* grounds from their histologic appearance. Pack and Ehrlich (*1*) hypothesize that this effect may be indirect, *i.e.*, due to suppression or cessation of ovarian function (radiation castration). In support of this hypothesis they adduce the evidence that a small dose of irradiation directed to the tumor site is usually ineffective and that larger doses directed to the lower abdomen as well are necessary for complete regression or satisfactory control of the tumor. They further support this hypothesis by pointing out the radio-resistance of desmoid tumors in males.

Lipschütz and his co-workers (*4, 5*) have produced connective-tissue tumors in male and female guinea pigs by administration of estrogen. These tumors regressed somewhat when estrogen administration was discontinued, and their formation was prevented by administration of

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testosterone, progesterone, or deoxycorticosterone along with the estrogen. It is noteworthy that these tumors were fibroblastic in origin, nonencapsulated, and had the ability to infiltrate skeletal muscle, but did not metastasize. Nadel (*6*) produced similar tumors in guinea pigs by similar methods. After detailed histologic study, he concluded that these estrogen-induced tumors were better classified as "desmoid tumors" rather than "fibroid tumors," the term used by Lipschütz.

There are two recorded instances of bioassay of desmoid tumors for hormonal activity. Geschickter and Lewis (*7*) report that a desmoid tumor of the abdominal wall contained 13,000 rat units of gonadotrophic substance per kilogram and contained no assayable estrogen. Unfortunately, they do not furnish data regarding the age or sex of the patient, whether the patient was or had been recently pregnant, nor how long the tumor had been present. Pack and Ehrlich (*1*) report that bioassay of one of the desmoid tumors in their series revealed it to contain 250 units per kilogram of *prolan A*. This tumor was removed from the abdominal wall of a 32-year-old white woman, *gravida I, para* II, 2½ years following her last previous delivery, the tumor having first been noted 6 months prior to operation and having grown to a size of 8 × 12 cm. Neither of these two reports of bioassay states what methods were used in arriving at the determinations.

Case History

C. A. E., an 18-year-old white, married woman, pregnant for the first time, was first seen at the Boston Lying-in Hospital on January 15, 1954. She was in the 17th week of gestation, and her expected date of confinement was May 11, 1954. Initial examination was not remarkable except for a 3-cm. mass in the left popliteal fossa. This was first noticed 6 weeks prior to conception, at which time it was 1 to 2 cm. in diameter and somewhat tender. The mass was fixed to the deeper tissues of the popliteal space, but the overlying skin was freely movable. She was referred to the Peter Bent Brigham Hospital, where a clinical diagnosis of Baker's cyst was made, and she was instructed to return after delivery for excision. By April 7, 1954, the mass had increased 2½ times in size and was interfering with the patient's walking. She was admitted to the Peter Bent Brigham Hospital on April 12, 1954. The mass was described as ovoid, slightly irregular, and firm though not hard. It did not transilluminate. The skin temperature over the mass was increased, but there was no discoloration. The peripheral pulses were strong and equal. X ray revealed a soft tissue mass displacing the leg muscles; the mass was seen to be extrinsic to bone, and there was no alteration in the appearance of the underlying bone. On April 14, 1954, an incisional biopsy was performed. The mass was found to be composed of firm, homogeneous, relatively avascular fibrous tissue. The diagnosis was desmoid tumor. It was decided to defer definitive surgical treatment until after delivery. On May 17, 1954, following a normal primiparous

labor, the patient was delivered of a normal, healthy female infant. She was transferred to the Peter Bent Brigham Hospital on May 24, 1954.

On May 29, 1954, the 12th postpartum day, an operation was performed. The tumor surrounded the popliteal artery and vein as well as the peroneal nerve; it was necessary to free the tumor from these structures by sharp dissection. Excised along with the tumor were the head of the gastrocnemius muscle, the popliteus, and the head of the semitendinosus. An ellipse of the skin overlying the tumor was excised in continuity with the tumor mass. At the completion of the operation the pulses in the extremity were strong and neuromuscular function was unimpaired. Recovery was uneventful, and the patient was discharged on June 19, 1954, with considerable improvement in the motion of the knee joint. No local recurrence was found on examination 1 year later.

Pathologic examination of the operative specimen revealed a tumor mass which weighed 410 gm. and which measured 8.5 × 13.5 cm. (fig. 1). The external surface was composed of fibro-fatty and muscular tissue. Attached to one surface was an ellipse of skin measuring 2.5 × 7 cm., in the center of which was a healed linear scar 3.5 cm. long. The tumor was ovoid, extremely hard and unresilient, and cut with considerable resistance. The cut surface was homogeneous, grayish-white, and glistening. Its texture was that of interlacing, loosely whorled fibers arranged in dense bundles with the characteristic appearance of watered silk, so frequently found in fibrous and muscular tumors. The tumor was not encapsulated and merged imperceptibly into the surrounding tissues; however, extension into the attached muscle was not grossly visible.

Microscopic examination revealed the tumor to be composed of spindle cells arranged in interlacing bundles (fig. 2). The spindle cells were extremely elongated with fusiform, sharply pointed nuclei, and inconspicuous cytoplasm. In most areas the nuclei were sparsely distributed and associated with the production of wavy, eosinophilic collagen fibers. The density of the fibers varied from a loose, reticular meshwork to a dense, hyalinized, brightly eosinophilic aggregation. In general the bundles of tumor cells were in the form of loosely interlaced bands rather than tight whorls. Mitotic activity was inconspicuous, nor was there any tendency to cellular pleomorphism. At its borders the tumor was sharply demarcated from the adjacent areolar connective tissue, but there was no microscopic evidence of a capsule. In one section taken to include both the tumor and the head of the gastrocnemius muscle, interdigitation of tumor tissue with adjacent muscle fibers for a short distance was observed, evidence of a limited ability of the tumor cells to infiltrate skeletal muscle. Preparations stained with Sudan III showed that the tumor cells did not contain stainable lipid. The final anatomic diagnosis was desmoid tumor of the popliteal fossa.

Bioassays

Forty-five grams of tumor tissue were vigorously extracted with physiologic saline to extract the proteinaceous hormones. Likewise, 45 grams

of tumor tissue were extracted with ether to extract the steroid hormones; this fraction was suspended in sesame oil. Estrogenic substances were bioassayed by the method of Hisaw, Velardo, and Goolsby (*8*) using 20 ovariectomized virgin albino rats 100 days old. The dosages of extract in experimental and control animals are given in table 1; 0.3 cc. of either extract is equivalent to 0.15 gm. of tumor. Progestational substances were bioassayed by the method of Velardo and Hisaw (*9*) using 24 ovariectomized virgin albino rats 100 days old. Bioassay for gonadotrophic, adrenocorticotrophic, and thyrotrophic substances was carried out by the method of Velardo (*10*) using 24 immature female rats 23 days old. Control data regarding the weights of organs and their microscopic appearances were obtained from nontreated rats of the same strain used in parallel experiments in this laboratory.

TABLE 1.—*Bioassay of desmoid tumor extract**

Number of animals and treatment	Uterine weight (*average in mg.*)	Adrenal weight (*average in mg.*)	Epithelium of vagina
Group I (18)			
Untreated controls	126	58. 7	Atrophic
Group II (18)			
0.05 μg. estradiol-17β	167	67. 2	Cornified
Group III (18)			
0.10 μg. estradiol-17β	237	71. 2	Cornified
Group IV (5)			
0.3 gm. tumor extract in saline	165	59. 8	Not cornified
Group V (5)			
0.9 gm. tumor extract in saline	202	64. 8	Not cornified
Group VI (5)			
0.3 gm. tumor extract in ether-oil	172	60. 0	Not cornified
Group VII (5)			
0.9 gm. tumor extract in ether-oil	200	66. 3	Not cornified

*In each instance the standard error of the mean was less than 10%.

The results of these bioassays for endocrine activity are as follows:

For estrogenic substances (see table 1).—Both saline and ether extracts of the tumor induced uterine growth in experimental animals. Control animals treated with 0.05 μg. of estradiol-17β had an increase in uterine weight of 32 percent. This corresponds with the results obtained by injecting 0.3 gm. of desmoid tumor extract either in saline (31% increase) or in ether suspended in sesame oil (35%). When a larger dose of estradiol-17β, 0.10 μg., was injected, the increase in uterine growth was 88 percent. Experimental animals treated with 0.9 gm. of desmoid tumor extract approached but did not reach this degree of response; both saline and ether extracts induced uterine growth increase in the order of 60 percent. It is of some significance that injections of estradiol-17β produced cornification of the vaginal epithelium at both 0.05 and 0.10 μg. dosage, but that desmoid tumor extract failed to produce cornification of vaginal epithelium irrespective of dosage or vehicle. There was no significant alteration in the weights of the adrenal glands in animals

treated with desmoid tumor extract when compared with normal, untreated controls, but significant increases were observed in animals treated with estradiol-17β.

For progestational substances.—Although there was an increase in weight of the traumatized cornu when compared with the nontraumatized cornu, this increase was of substantially the same magnitude in untreated controls as in animals treated with saline or ether extracts of the desmoid tumor in doses of 0.3 and 0.9 gm. Animals treated with 0.25 or 0.50 mg. of progesterone exhibited a significant percentage of weight increment in the traumatized cornu over normal controls, and deciduomas were formed at the site of trauma. In no case did desmoid tumor extract elicit the formation of decidua.

For gonadotrophic substances.—When compared with normal, untreated control animals, extracts of the desmoid tumor in doses of 0.15 or 0.45 gm. failed to induce any significant increase in the weight of the uteri, ovaries, adrenals, or thyroids. Microscopic examination of the ovaries revealed very small follicles in both normal animals and animals treated with tumor extract. The vaginae of both controls and extract-injected animals remained closed. Control animals given HCG, follicle-stimulating hormone, and LH, respectively, responded characteristically to gonadotrophic substances.

From the above data, it is evident that the extracts of the desmoid tumor contained no progestational or gonadotrophic substances. However, there is evidence that a substance which promotes uterine growth but does not induce cornification of vaginal epithelium was present in the tumor. Such a substance has been described by Cohen and Bates (*11*) in the nonketonic, phenolic fraction of pregnant mares' urine extracted with toluene. The "neutral" fraction remaining in the toluene increased uterine weight in hypophysectomized, ovariectomized, or adrenalectomized rats. This fraction did not induce cornification of the vaginal epithelium when administered alone, but it augmented the effects of estrogenic substances when administered concomitantly.

Following this initial bioassay, additional studies were performed.[2] Ninety-two grams of the tumor were extracted with alcohol and then with toluene. The toluene extract was separated into neutral and phenolic fractions which were evaporated and stored in 95 percent ethyl alcohol.

Utilizing these fractions, further bioassay was performed. The fractions were each tested for estrogenic activity by the method of Hisaw *et al.* (*8*) using 20 ovariectomized virgin albino female rats weighing about 200 gm. each. The dosage for each group and the average uterine weight, average adrenal weight, and appearance of the vaginal epithelium are presented in table 2. There was no histologic evidence of estrogenic effect on uterine growth, nor was any adrenal atrophy demonstrable. No significant differences in uterine or adrenal weights were noted in the experimental

[2] The extraction was graciously performed by Dr. Olive Watkins Smith, Fearing Laboratory, Free Hospital for Women, Brookline, Mass.

TABLE 2.—*Bioassay of neutral and phenolic fractions for estrogenic activity**

Number of animals and treatment	Uterine weight (*average in mg.*)	Adrenal weight (*average in mg.*)	Cornification of vaginal epithelium
Group I Untreated controls	115. 5	59. 9	None
Group II (5) 0.1 cc. phenolic fraction	122. 1	63. 7	None
Group III (5) 0.2 cc. phenolic fraction	117. 4	62. 1	None
Group IV (5) 0.25 cc. neutral fraction	106. 8	64. 3	None
Group V (5) 0.50 cc. neutral fraction	126. 0	54. 4	None

*In each instance the standard error of the mean was less than 10%.

animals when compared with the controls, nor was there any cornification of the vaginal epithelium.

It was thus determined that neither the phenolic nor the neutral fraction possessed any detectable estrogenic substances *per se*. To determine whether either fraction contained substances which would augment or enhance the effects of concomitantly administered estrogens, a further assay was performed. Thirty-one virgin albino rats, weighing about 200 gm., were subjected to bilateral ovariectomy. One week later each animal was injected subcutaneously daily for 3 days with estradiol-17β either alone or in combination with one of the tumor-extract fractions. The animals were necropsied on the fourth day and the uteri, vaginae, and adrenal glands weighed and examined histologically as above. The dosage of estradiol, combination with and dosage of tumor

TABLE 3.—*Bioassay of neutral and phenolic fractions for estrogen-augmenting activity**

Number of animals and treatment	Uterine weight (*average in mg.*)	Adrenal weight (*average in mg.*)	Cornification of vaginal epithelium
Group I (7) 0.05 μg. estradiol-17β	149. 2	61. 0	Slight
Group II (4) 0.10 μg. estradiol-17β	222. 5	57. 6	Moderate
Group III (5) 0.05 μg. estradiol-17β 0.1 cc. phenolic fraction	144. 9	68. 5	Marked
Group IV (5) 0.05 μg. estradiol-17β 0.2 cc. phenolic fraction	150. 0	60. 0	Very marked
Group V (5) 0.05 μg. estradiol-17β 0.25 cc. neutral fraction	169. 0	59. 8	Slight
Group VI (5) 0.05 μg. estradiol-17β 0.50 cc. neutral fraction	173. 1	58. 3	Slight

*In each instance the standard error of the mean was less than 10%.

extract, and the average uterine weight, adrenal weight, and observations on the cornification of vaginal epithelium are presented in table 3. It is calculated that both 0.1 cc. of the phenolic fraction and 0.25 cc. of the neutral fraction are equivalent to 0.46 gm. of the original desmoid tumor.

From the data in table 3 it is apparent that the phenolic fraction serves to augment estrogenic stimulation of the cornification of vaginal epithelium, but has no appreciable enhancing effect upon uterine growth induced by estrogen stimulation. The opposite effect is found in the neutral fraction which slightly augments estrogen-induced uterine growth but has no appreciable effect upon cornification of vaginal epithelium. The separation of the tumor extract into neutral and phenolic fractions demonstrates that estrogen effects upon end organs are dissociable by the assay methods used.

There was no opportunity to prepare control extracts from the small amount of areolar connective tissue which surrounded the excised desmoid tumor, and it is not possible to state with certainty whether the estrogen-augmenting effects observed are characteristic of the tumor or are a nonspecific property of any tissue removed from a woman 12 days postpartum.

Summary

1) The occurrence of a desmoid tumor in the popliteal fossa of a pregnant woman, its rapid growth during pregnancy, and its surgical removal 12 days postpartum are reported.

2) Saline and ether extracts of this tumor stimulated uterine growth but did not produce cornification of vaginal epithelium in rats. The tumor did not contain assayable estrogenic, progestational, gonadotrophic, adrenotrophic, or thyrotrophic substances.

3) The neutral fraction contained substances which augmented estrogen-induced uterine growth. The toluene fraction contained substances which augmented estrogen-induced cornification of vaginal epithelium.

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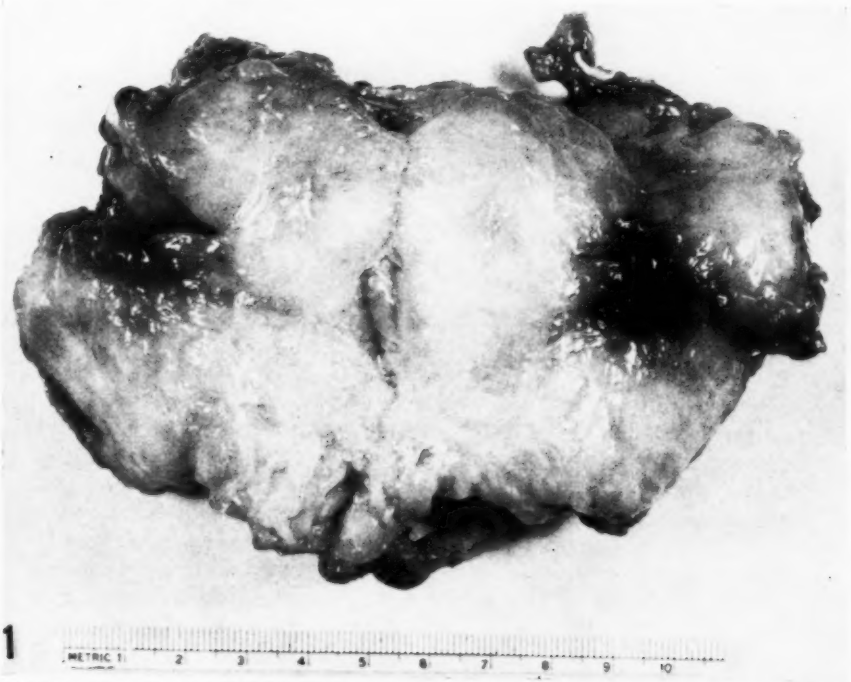
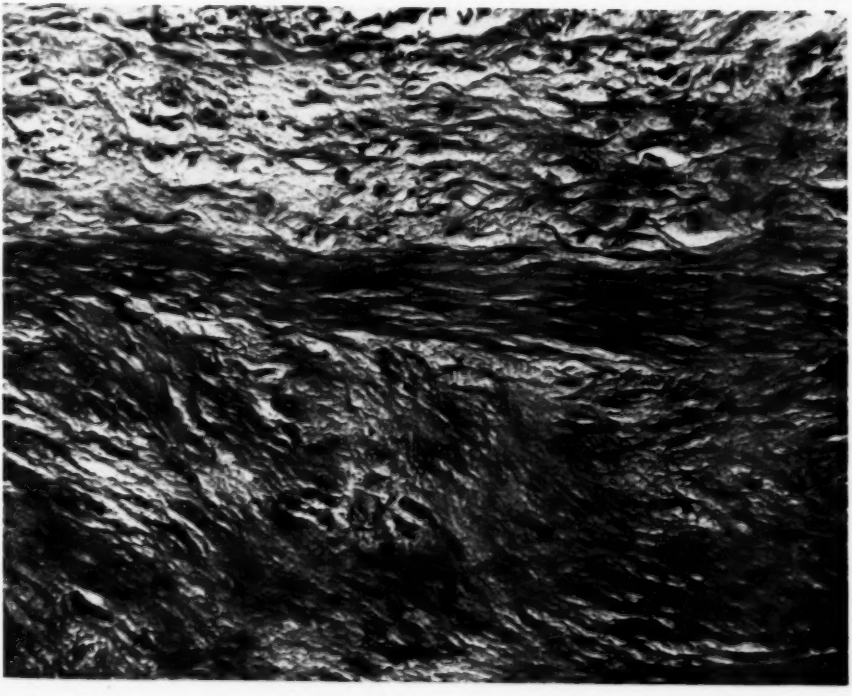
PLATE 42

FIGURE 1.—Gross photograph showing the dense, fibrous appearance of the cut surface of the tumor. The whorls and bundles are not compactly arranged.

FIGURE 2.—Sparsely nucleated tissue traversed by bands of packed collagen fibers comprise the substance of the tumor. Hematoxylin and eosin. × 210





352471—55——19

A Preliminary Study of Nuclear Size in Tissues from Tumorous and Nontumorous Mice[1,2]

FREDERICK H. KASTEN, *Biological Station, Roswell Park Memorial Institute, Springville, N. Y.*

In recent years there have been several studies comparing the size of nuclei of tumor and non-tumor cells (*1–3*). Other investigators have found it of interest to determine the stability of nuclear sizes in differentiated cells from tumor-bearing animals. A few reports in the literature suggest that nuclear sizes are affected in tumor-bearing animals (*4–7*). These studies merit further investigation, and the report presented here constitutes a preliminary study of this problem. As to the method of approach, a decision had to be made whether to obtain a large number of measurements from a few animals or to use a larger sampling of animals with a smaller number of measurements from each animal. It was decided, somewhat arbitrarily, to select the first method. The limitations of the conclusions in this preliminary study are realized by the writer.

Materials and Methods

Four mice bearing spontaneous tumors were etherized and then sacrificed by decapitation. Litter mates, and in two cases mice of comparable strain, age, and sex that were not litter mates, were used as control animals. In addition, a transplantable spindle-cell neoplasm (*8*) was inoculated into several 6-week-old mice of the PBR strain.[3] These mice developed tumors ranging from 0.2 to 4.5 grams and were later sacrificed along with their controls. Table 1 describes the animals and the tumors used.

Whole adrenal glands and pieces of liver and kidney (2–3 mm. and 2 mm., respectively) were removed and fixed in 50-percent formalin. Tissue from these organs was chosen because the nuclear sizes of cells from such tissue are easily estimated. Sections of tissue to be compared were mounted on the same slide and stained by the Feulgen technique (*9*). By use of a calibrated micrometer disc the nuclear diameters could be measured at a magnification of ×360, read to the nearest 0.5μ, and approximated to 0.25μ. Nuclei from the tissues studied were usually round, but in a small number of cases, slightly aspherical nuclei appeared.

[1] Received for publication January 27, 1955.

[2] A preliminary report of this work was published in Proc. Am. Assoc. Cancer Res. 2: 28, 1955.

[3] The writer acknowledges the assistance of Mr. Harold Woodworth.

TABLE 1.—*Description of mice and tumors used**

Animal	Strain	Sex	Age at sacrifice (*months*)	Tumor		
				Present	Diagnosis†	Weight (*gm.*)
A.....	C3H	female	7	yes	spontaneous mammary adenocarcinoma (two tumors)	10. 5 6. 4
control.....	C3H	female	8	no	—	—
B.....	5 Prunt	female	7	yes	spontaneous mammary adenocarcinoma	4. 6
control.....	5 Prunt	female	7	no	—	—
C.....	C3H	female	12	yes	spontaneous mammary adenosarcoma (collision?)‡	22. 0
control.....	C3H	female	11	no	—	—
D.....	C3H	female	8	yes	spontaneous mammary adenocarcinoma	3. 0
control.....	C3H	female	8	no	—	—
5-Day transplant.	PBR	female	1. 5	yes	spindle-cell neoplasm§	0. 2
14-Day transplant	PBR	male	2	yes	spindle-cell neoplasm§	0. 5
20-Day transplant	PBR	male	1. 5	yes	spindle-cell neoplasm§	4. 5
Transplant control	PBR	male	1. 5	no	—	—

*These mice were obtained from the breeding stocks of Dr. L. C. Strong.
†The diagnoses were made by Mr. Hugh O'Neill.
‡Probably an adenocarcinoma of the mammary gland with an active stroma.
§Described in detail by Bagshaw and Strong (*8*).

In the latter group, the major and minor axes were averaged to give the mean diameter. Nuclear volumes were computed by considering the round or slightly aspherical nuclei to be spheres and letting the nuclear volumes be a function of the radii cubed.

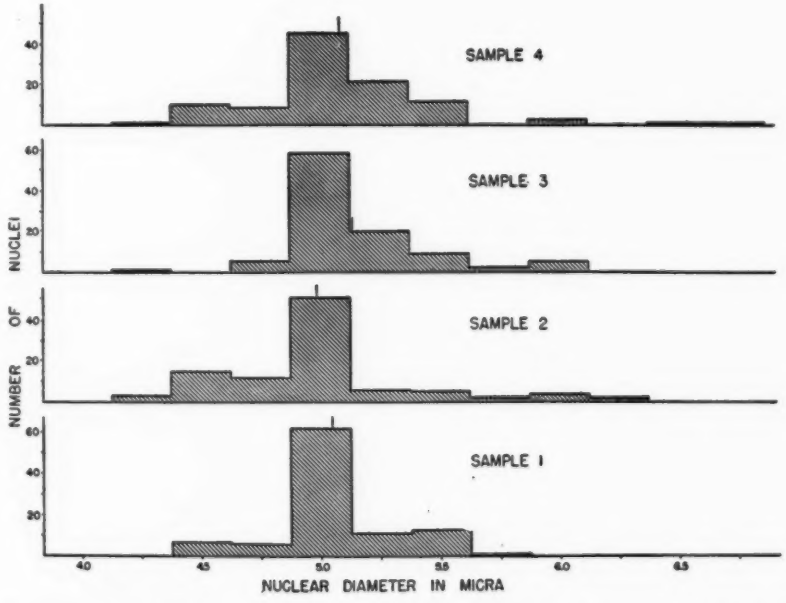
About 100 interphase nuclei were measured in each sample from the adrenal gland (entire zona fasciculata except for marginal regions) and the kidney (2 mm. of tissue from the craniad region, mostly cortex). Statistical evaluations of possible differences were made by the Student *t* test (*10*). In each sample from the liver, 200 interphase parenchyma nuclei were measured. Because of the complication of polymodal classes in the liver, the conventional statistical analyses were avoided. Instead, modal values were obtained for Class 1 and Class 2 nuclei[4] using a method described in detail by Salvatore (*11*). Classes with higher nuclear volume than Class 2 were not studied, since in many cases these classes contained

[4] The terms Class 1 and Class 2 nuclei are used here to designate the first two modal nuclear-volume groups usually observed in livers of adult mice.

few nuclei whose distributions were irregular. Graphic comparisons were also made using probability paper (*12, 13*). In order to evaluate correctly possible differences between the sizes of nuclei in tissue from tumorous and nontumorous mice, a study was made of the variations in nuclear size among different nontumorous mice of a given strain, age, and sex. Approximately 7,000 nuclei were measured in the work reported here.

Results

Adrenal gland.—The frequency histogram in text-figure 1 illustrates the variation in diameters of nuclei from adrenal-gland tissue of several nontumorous mice of the same strain, age, and sex. The mean values of nuclear diameter range from 5.0 to 5.1μ. The data are presented in table 2. Table 3 compares nuclear sizes in the adrenal glands of four tumor-bearing animals (A–D) and their respective controls. There are no consistent differences in these comparisons. Further data are included concerning nuclear sizes in adrenal glands from three mice bearing spindle-cell neoplasms and from their controls. The mean diameters for these tumorous mice show a significant increase over those of the control mouse. When a second series of measurements was made on the control tissue, using the same slide, similar results were obtained. The frequency distributions for these data are shown in text-figure 2.



TEXT-FIGURE 1.—Nuclear-size distributions from adrenal glands (zona fasciculata) of four different nontumorous adult mice (strain C3H virgin females, 4 months old). Vertical line in each group is the mean value.

TABLE 2.—*Nuclear-size data from a group of normal mice of comparable strain, age, and sex**

Animal	Mean nuclear diameter ± standard deviation†		Liver modal values‡		Class 2 / Class 1
	Adrenal cortex	Kidney	Class 1	Class 2	
1	5.05 (0.29)	5.00 (0.25)	24.7	49.4	2.0
2	4.99 (.40)	4.95 (.32)	25.5	47.5	1.9
3	5.14 (.29)	4.97 (.32)	25.9	47.2	1.8
4	5.08 (.40)	4.99 (.49)	24.7	46.3	1.9
5	—	—	30.5	46.5	1.5

*Four-month-old strain C3H virgin females.

†Means are averaged from 100 measurements in each group, expressed in μ.

‡Modal values obtained from formula given by Salvatore (*11*) and expressed as radius cubed. Each frequency distribution contains 200 measurements.

TABLE 3.—*Nuclear-size data from a group of tumorous mice and their controls*

Animal	Mean nuclear diameter ± standard deviation*		Liver modal values†		Class 2 / Class 1
	Adrenal cortex	Kidney	Class 1	Class 2	
A	5.25‡ (0.44)	5.26 (0.40)	26.7	47.3	1.8
control	4.77 (.28)	5.30 (.42)	28.0	50.1	1.8
B	4.67 (.32)	5.01‡ (.41)	26.4	49.0	1.85
control	4.73 (.28)	4.78 (.42)	30.6	45.9	1.5
C	5.09 (.50)	5.11‡ (.47)	14.0	49.6	3.5
control	5.10 (.36)	5.35 (.44)	17.4	49.0	2.8
D	5.03‡ (.25)	5.06 (.37)	28.0	49.3	1.8
			30.2	49.0	1.6
control	4.77 (.28)	5.12 (.34)	28.0	50.1	1.8
			25.4	49.4	1.9
5-Day transplant	5.13‡ (.43)	5.62‡ (.52)	26.6	50.4	1.9
14-Day transplant	5.29‡ (.36)	5.50‡ (.54)	26.7	49.0	1.8
20-Day transplant	5.39‡ (.43)	5.40‡ (.56)	25.3	52.5	2.1
Transplant control	4.74 (.33)	4.69 (.31)	25.1	49.5	2.0
	4.87 (.30)				

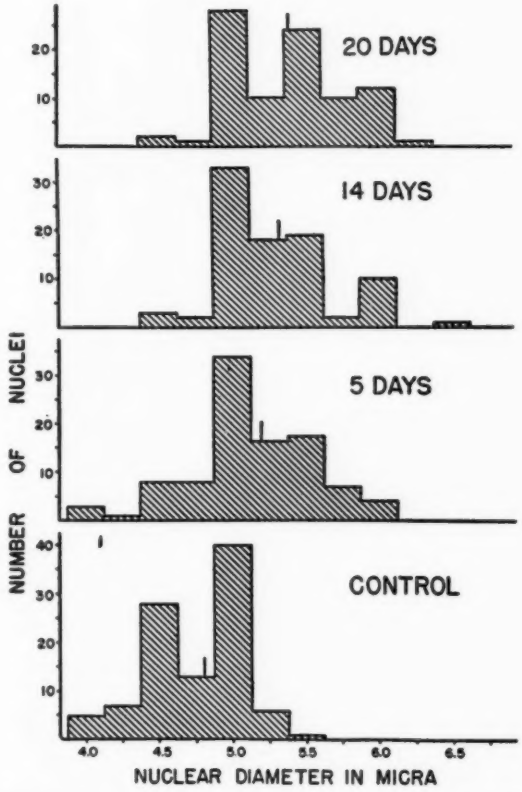
*Means are averaged from 88 to 100 measurements in each group, expressed in μ.

†Modal values obtained from formula given by Salvatore (*11*) and expressed as radius cubed. Each frequency distribution comprises 200 measurements.

‡Significantly different from control animal at 1% level as calculated by Student t method given by Hoel (*10*).

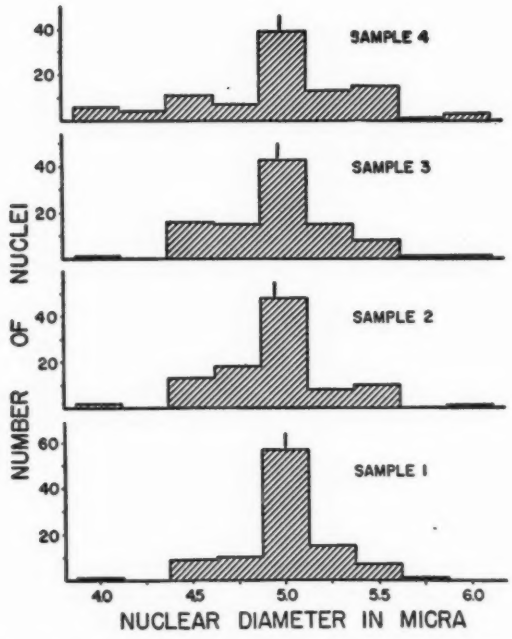
Kidney.—Frequency histograms of nuclear-diameter measurements of kidney tissue from four normal mice of similar strain, age, and sex are shown in text-figure 3. The mean values of nuclear diameter are 5.0μ for each sample. Table 3 shows that there are no consistent changes in mice bearing spontaneous tumors. However, nuclear diameters in the three mice bearing transplanted spindle-cell neoplasms are 0.7 to 0.9μ larger than the diameters in the control mouse. A second slide from the control kidney was examined and similar results were obtained. These differences are shown in text-figure 4.

Liver.—Individual variations in nuclear volumes in liver tissue from normal mice are summarized in table 2. Modal values for Class 1 nuclei range from 25 to 30.5μ^3. Class 2 modal values range from 46 to 49μ^3. The average ratio of modal values for Class 2/Class 1 nuclei (5 samples) is 1.8, a value slightly less than the theoretical 2/1 ratio expected in a true polyploid relation. With these data in mind, the results of karyometric measurements in liver may be compared for the tumor-bearing



TEXT-FIGURE 2.—Comparison of frequency distributions of adrenal cortical nuclei from mice with spindle-cell neoplasms and the control mouse. Vertical line in each group is the mean value.

and control groups. Table 3 shows the modal values for Class 1 and Class 2 nuclei. In both classes, there is no appreciable difference between control and tumor-bearing mice. It is also noted that there are no differences in mice bearing spindle-cell neoplasms and the control mouse. However, if one plots these data on probability paper (text-fig. 5), it is seen that in tumorous animals a greater number of large nuclei exist than in the control animal.

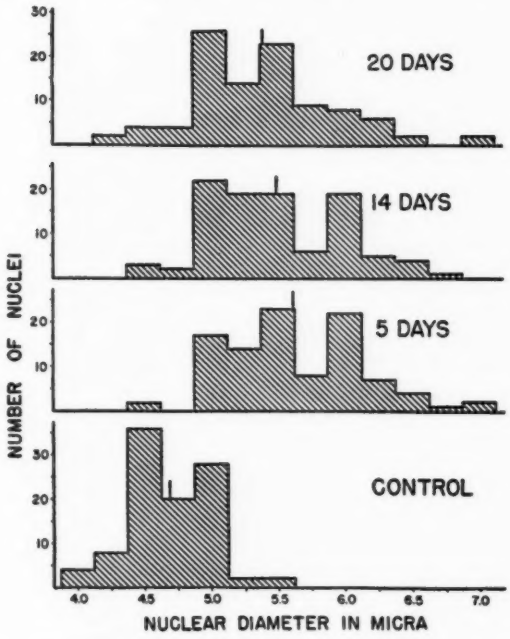


TEXT-FIGURE 3.—Nuclear-size distributions from kidneys of four different non-tumorous adult mice (strain C3H virgin females, 4 months old). Vertical line in each group is the mean value. Note uniformity in sample distributions.

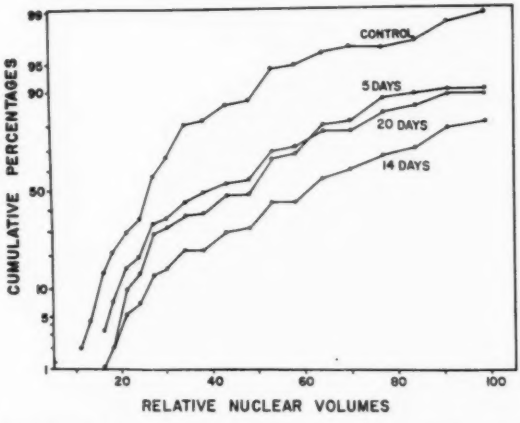
Discussion

Microscopic measurements of nuclear sizes are subject to certain errors caused in part by the dimensions of nuclei and in part by the preparation and nature of the tissue (*14*). A few important points will be mentioned here. Karyometric analyses of cell populations have often been made without considering the variability in samplings from a given tissue. It is obvious that unless one knows the degree of variability in a given tissue or among several individuals in a given group, it is difficult to evaluate differences between two or more groups. In the present study, it was noted that populations of nuclei from adrenal cortex, kidney, and liver are very similar among normal individual mice of a given group. However, nuclear-size classes in liver are often localized (see figs. 1 and 2). Jacobj (*15*) made a similar observation. Since nuclear sizes often run parallel to changes in chromosome number in a given tissue of a species (*16*), another source of variation may follow deviations from the diploid chromosome number in somatic tissues [summarized in (*17*)].

It is well known that the choice of fixative, as well as the staining treatment of the tissue, affects nuclear sizes seen in a final preparation (*18*). Hertwig (*19*) found that reproducible results could be obtained in measurements of nuclei fixed identically. In the present work, all



TEXT-FIGURE 4.—Comparison of frequency distributions of kidney nuclei from mice with spindle-cell neoplasms and the control mouse. Vertical line in each group is the mean value.



TEXT-FIGURE 5.—Comparison of cumulative frequency distributions of nuclear volumes from livers of mice bearing spindle-cell neoplasms and the control mouse. Tumor-bearing mice have a greater number of large nuclei than the control mouse. Plot is on probability paper.

tissues of approximately the same thickness were fixed in 50-percent formalin for the same length of time. Subsequent washing, dehydration, clearing, and paraffin infiltration were made as standard as possible. Measurements of nuclear sizes were made from sections which had been mounted on the same slide and which were then treated identically. It is therefore felt that this potential source of error was kept at a minimum.

Another potential source of error involves the fraction of nuclear fragments present in a tissue section. Such measured fragments are said to give an erroneous frequency distribution (*14*). This source of error is probably of minor importance when spherical nuclei are measured, since nuclear diameters are measurable even when small portions of nuclei are fragmented away from their diameters.

There are a few reports in the literature concerning the possible effects of tumors on nuclear sizes in nontumorous tissues. According to Wermel and Scherschulskaja (*4*), nuclei of noncancerous cells surrounding a skin cancer are larger than the corresponding nuclei from normal tissue. A group of Italian workers reported various changes in nuclear sizes of adrenal glands (*6*), kidneys (*7*), and livers (*5*) of mice bearing transplanted mammary carcinoma. The adrenal cortical nuclei were said to be smaller in tumorous mice, while nuclei from the adrenal medulla were found to be larger than the corresponding normal nuclei. Kidney nuclei were also found to be larger in tumorous mice. The number of large liver nuclei was reported to increase progressively with tumor growth.

The present work, where four spontaneous tumors and a transplantable tumor were used, gave results which were not entirely clear-cut. In the case of the spontaneous-tumor group, there were no consistent changes in any of the organs studied although some statistically significant differences were measurable. In the case of the transplanted-tumor group, nuclear measurements from the three mice bearing tumors differed consistently from measurements in the control mouse. Adrenal cortical nuclei and kidney nuclei were significantly larger in the tumorous mice than in the control. In the adrenal cortex there is a shift of mean diameter from 4.8μ in the control mouse to 5.4μ in the 20-day tumorous mouse. The actual change of volume is best given by the cube of the ratio of the diameters: $\sim\left(\frac{5.4}{4.8}\right)^3 \sim 1.4$. For the kidney, there are similar volume changes given by the ratio: $\sim\left(\frac{5.5}{4.7}\right)^3 \sim 1.6$. If these measurements are true samplings from their respective nuclear populations, the average nuclear volumes have increased approximately 50 percent in both cases. These nuclear-volume changes might result from differences in water content, nuclear proteins, or perhaps deoxyribonucleic acid. Chemical analyses or cytophotometric measurements might be used to further analyze this problem. If the changes measured are real, it would be of interest to learn whether nuclei in other organs show similar effects. It would also be of special significance to learn whether the nuclear-volume changes reflect cellular responses to a general pathologic condition of the animal or perhaps a specific response to a tumorous condition.

In addition to the changes measured in adrenal glands and kidneys, there is also a greater frequency of large nuclei in livers of mice with transplanted tumors as compared with the control. Since the mitotic index is very low in all livers studied from both tumorous and control mice, the increased number of large liver nuclei probably does not result from a mitotic phenomenon. The influence of the pituitary growth hormone on the production of higher nuclear classes in mice (*20*) may be a factor here. It is noted also that liver nuclear sizes in the tumorous group do not differ from those in the control mouse.

Since one animal was used as a control in the transplanted-tumor group, it is possible that the differences reported were due entirely to chance. Until the changes reported here for the transplanted-tumor group are verified, it would be premature to speculate on the reason(s) for the difference between the spontaneous-tumor group and the transplanted-tumor group.

Summary

1) Approximately 7,000 nuclei were measured from adrenal glands (zona fasciculata), kidneys, and livers of tumorous mice and control mice to evaluate possible differences in nuclear sizes. Four spontaneous tumors and a transplanted spindle-cell neoplasm were used.

2) Individual nuclear-size variations in these tissues were studied in four mice of the same strain, age, and sex in order to assess possible differences between tumorous and nontumorous mice.

3) No consistent changes were observed in mice with spontaneous mammary tumors. Mice bearing transplanted tumors showed significant increases in nuclear size in adrenal glands and kidneys compared with nuclear size in the control mouse. These increased sizes corresponded to nuclear volume increases of approximately 50 percent. In addition, there was an increase in frequency of large nuclei in livers of mice bearing transplanted tumors.

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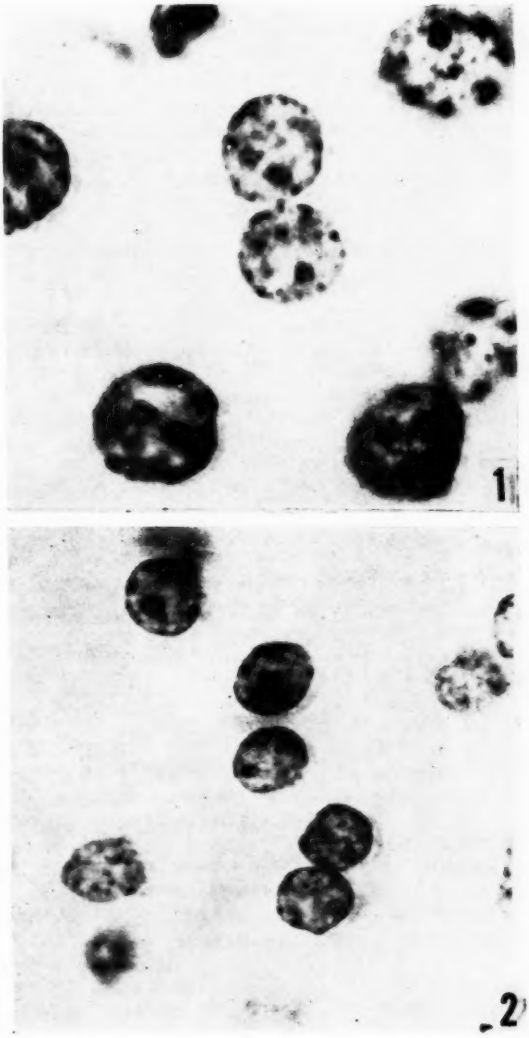
PLATE 43

FIGURE 1.—Photomicrograph of liver-parenchyma nuclei from a 2-month-old mouse. Note localization of large nuclei in the field. Tissue section stained by the Feulgen technique. × 1,600

FIGURE 2.—Photomicrograph of liver-parenchyma nuclei from same section shown in figure 1. Note localization of small nuclei in the field. These localizations may contribute to sampling errors when measuring nuclear sizes in liver. × 1,600



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ANNOUNCEMENTS

Gastrointestinal Cancer Conference

The Proceedings of the Sixth National Gastrointestinal Cancer Conference, sponsored by the National Cancer Institute, and held in New York, April 1955, appears in the October issue of Gastroenterology. Proceedings of the previous five Conferences can be found in the Journal of the National Cancer Institute, volume 1, page 423; volume 5, page 311; volume 7, page 299; volume 10, page 297; and volume 13, page 927.

Rhesus Monkeys for Medical Research and Production of Antipoliomyelitis Vaccine

As a result of negotiations, the United States Mission to India and the United States Embassy in India have secured the agreement of the Government of India to permit the continued exportation of rhesus monkeys for medical research and production of antipoliomyelitis vaccine. All needs must be certified by the United States Government through the Public Health Service.

Information concerning Public Health Service procedure and Certificate of Need forms may be secured from: *National Institutes of Health, Room 1012, Building T-6, Bethesda 14, Maryland (Telephone: Oliver 6-4000, Extension 2748, Miss Parent).*

Medical Research Fellowships

The Division of Medical Sciences of the National Academy of Sciences-National Research Council is accepting applications for postdoctoral research fellowships for 1956–1957. These awards are designed to offer research experience for promising individuals who look forward to investigative careers, and not to provide practical experience in the clinical field. Ordinarily fellowships are not granted to persons over 35 years of age. Programs in the field of cancer are:

Fellowships in Cancer Research.—Awarded by the American Cancer Society on recommendation of the Committee on Growth of the Division of Medical Sciences. Awards are available for study in all branches of the biological, chemical, and physical sciences and of clinical investigation applicable to the study of growth, typical or malignant. Citizens of the United States are eligible.

British-American Exchange Fellowships in Cancer Research.—Also awarded by the American Cancer Society upon recommendation by the Committee on Growth. They are offered to citizens of the United States for advanced study in Great Britain in specialized fields pertaining to the problem of growth. Similar fellowships are awarded by the British Empire Cancer Campaign to young British scientists for research in the United States.

Programs offered in other fields are: Fellowships in the Medical Sciences, Tuberculosis, and Radiological Research.

Applications for 1956–1957 under any of these programs must be postmarked on or before December 1, 1955. Fellowships are awarded in the early spring. Complete details and application blanks may be obtained from the *Fellowship Office, National Academy of Sciences-National Research Council, 2101 Constitution Avenue, N.W., Washington 25, D. C.*

Grants for Scholars in Cancer Research

The Committee on Growth of the National Academy-Research Council invites applications for Grants for Scholars in Cancer Research offered by the American Cancer Society. The purpose of these grants is to assist institutions in the support of young scientists during the critical early period of their careers as independent investigators. Grants are awarded for 3 years ($6,000 per year) with renewal for 2 additional years unless there are compelling reasons to the contrary. *Applications should be submitted by institutions on behalf of candidates not later than 1 January.*

Application blanks may be obtained from the Executive Secretary, Committee on Growth, National Academy-Research Council, 2101 Constitution Avenue NW., Washington 25, D. C.

U. S. GOVERNMENT PRINTING OFFICE: 1955

The Journal of the National Cancer Institute is published bimonthly by the National Cancer Institute of the National Institutes of Health, Bethesda, Md. Six issues, beginning with number 1 in August, comprise each yearly volume. The Journal publishes original observations and investigations in laboratory and clinical research in cancer. Manuscripts are considered for publication with the understanding that they have not been published previously.

Publication is not limited to members of the National Cancer Institute or to those whose work is supported by Government funds. Manuscripts should be submitted to the Director, National Cancer Institute, Bethesda 14, Md.

Suggestions to Authors

1. Submit an original and two carbon copies of the manuscript. Follow the style of a recent copy of the Journal, especially for illustrations, references, and tables. The latest edition of Webster's International Dictionary is followed in matters of spelling, capitalization, punctuation, compounds, and hyphenation. Current medical dictionaries and Chemical Abstracts are usually followed for medical and chemical terms. Type all material double spaced.

2. In the by-line, include the name of the institution with which each author is connected. Official title or academic degree is optional.

3. Begin each page with a new paragraph; *i.e.*, each page should contain one or more complete paragraphs, and no paragraph should carry over from one page to the next unless it is longer than a full page. Do not begin a new paragraph on a page carrying a run-over paragraph. This is a requirement of the Government Printing Office.

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CONTENTS

JOURNAL OF THE NATIONAL CANCER INSTITUTE • VOL. 16 · NO. 2 · OCTOBER 1955

Page

Regulation of glucose utilization in tumors by a stress-modified insulin : anti-insulin system. *Mark Woods, Jehu Hunter,* and *Dean Burk* 351

Differential effects of temperature on the growth of certain transplanted tumors in strain DBA mice. *Jehu C. Hunter* 405

A phase-contrast microscope study of free cells native to the peritoneal fluid of DBA/2 mice. *Marie D. Felix* and *A. J. Dalton* 415

Experimental studies in metal cancerigenesis. VII. Tissue reactions to parenterally introduced powdered metallic chromium and chromite ore. *W. C. Hueper* 447

Lack of carcinogenicity of lyophilized *Schistosoma* in mice. *Michael B. Shimkin, Piero O. Mustacchi, Eloise B. Cram,* and *Willard H. Wright* 471

The effects of tyrosine and phenylalanine on the synthesis of pigment in melanocytes of embryonic chick skin cultured *in vitro*. *John W. Saunders, Jr., Walter C. Quevedo, Louis Pierro,* and *Floyd E. Morbeck* 475

Tumors induced by 2-acetylaminofluorene in Buffalo-strain rats ingesting different levels of riboflavine. *Harold P. Morris, Billie P. Wagner,* and *Clarence A. Velat* 489

Neoplasms in the C3H strain and in F_1 hybrid mice of two crosses following introduction of extracts and filtrates of leukemic tissues. *L. W. Law, Thelma B. Dunn,* and *Peter J. Boyle* 495

The *p*H of rat tumors measured *in vivo*. *M. Eden, B. Haines,* and *H. Kahler* .. 541

The nutrition of animal tissues cultivated *in vitro*. I. A survey of natural materials as supplements to synthetic medium 199. *Joseph F. Morgan, M. Elizabeth Campbell,* and *Helen J. Morton* 557

Desmoid tumor of the popliteal space occurring during pregnancy: report of a case with bioassays. *William B. Ober, Joseph T. Velardo, Robert T. Greene,* and *Richard J. Taylor* 569

A preliminary study of nuclear size in tissues from tumorous and nontumorous mice. *Frederick H. Kasten* 579

Announcements 591